VOLUME 37 FEBRUARY 1959 NUMBER 2

Canadian Journal of Biochemistry and Physiology

Editor: K. A. C. ELLIOTT

Associate Editors:

B. D. BURNS, *McGill University*
L.-P. DUGAL, *University of Ottawa*
G. KROTKOV, *Queen's University*
A. G. McCALLA, *University of Alberta*
J. A. McCARTER, *Dalhousie University*
M. NICKERSON, *University of Manitoba*
H. E. RAWLINSON, *University of Alberta*
R. J. ROSSITER, *University of Western Ontario*
A. E. WILHELMI, *Emory University*

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Biochemistry and Physiology

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY and five other journals devoted to the publication, in English or French, of the results of original scientific research. *La Revue accepte des travaux originaux en biochimie, physiologie, pharmacologie, et sujets connexes.*

Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies. The Canadian Physiological Society has chosen the Canadian Journal of Biochemistry and Physiology as its official journal for the publication of scientific papers.

EDITORIAL BOARD

Representatives of the National Research Council

A. GAUTHIER, *University of Montreal*
R. B. MILLER, *University of Alberta*
H. G. THODE, *McMaster University*
D. L. THOMSON, *McGill University*

Editors of the Journals

D. L. BAILEY, *University of Toronto*
T. W. M. CAMERON, *Macdonald College*
H. E. DUCKWORTH, *McMaster University*
K. A. C. ELLIOTT, *Montreal Neurological Institute*
LÉO MARION, *National Research Council*
R. G. E. MURRAY, *University of Western Ontario*

Representatives of Societies

D. L. BAILEY, *University of Toronto*
Royal Society of Canada
T. W. M. CAMERON, *Macdonald College*
Royal Society of Canada
H. E. DUCKWORTH, *McMaster University*
Royal Society of Canada
Canadian Association of Physicists
K. A. C. ELLIOTT, *Montreal Neurological Institute*
Canadian Physiological Society
P. R. GENDRON, *University of Ottawa*
Chemical Institute of Canada
R. G. E. MURRAY, *University of Western Ontario*
Canadian Society of Microbiologists
T. THORVALDSON, *University of Saskatchewan*
Royal Society of Canada

Ex officio

LÉO MARION (Editor-in-Chief), *National Research Council*
J. B. MARSHALL, Division of Administration and Awards, *National Research Council*

Manuscripts for publication should be submitted to Dr. K. A. C. Elliott, Editor, Canadian Journal of Biochemistry and Physiology, Montreal Neurological Institute, 3801 University Street, Montreal, Canada.

(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover).)

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration and Awards, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration and Awards, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Monthly	$ 9.00 a year
Canadian Journal of Botany	Bimonthly	$ 6.00
Canadian Journal of Chemistry	Monthly	$12.00
Canadian Journal of Microbiology	Bimonthly	$ 6.00
Canadian Journal of Physics	Monthly	$ 9.00
Canadian Journal of Zoology	Bimonthly	$ 5.00

The price of regular single numbers of all journals is $2.00.

Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 37 FEBRUARY 1959 NUMBER 2

SOME EVIDENCE CONCERNING THE MODE OF BINDING OF HISTAMINE[1]

FRED J. MCILREATH, JR.

Abstract

The release of histamine from dog liver particles and isolated perfused cat paws was induced by various histamine liberators in both isotonic and hypertonic solutions of sucrose and mannitol. When any of several base-type histamine liberators were employed, significantly less histamine was discharged from the preparations in 1.2 *M* solutions than in isotonic solutions (0.32 *M*). No significant differences were found when surface-active compounds were used as liberators.

Introduction

In spite of the large volume of work being done with regard to the release of histamine, more evidence is needed concerning its mode of binding in the tissues. The work of Riley and West (20) has, I think, firmly established that much of the histamine in the body is contained in the tissue mast cells. It appears that the histamine discharged by the so-called "histamine liberators", as well as the anaphylactic reaction is derived from these cells since both the histamine and mast cell content of various tissues are decreased by the action of these liberators (17, 20), and by the antigen–antibody reaction (18).

When homogenates are prepared from tissues rich in mast cells, large quantities of histamine remain in the particulate phase (7, 9, 14, 23). This histamine is associated with the large granule fraction (2), and can be released by incubation with histamine liberators (6, 15). However, the addition of the specific antigen, which results in the discharge of histamine from sensitized tissues (16, 18), has thus far proved ineffective in releasing it from the homogenates (7, 15).

The fact that histamine can be released by certain purely physical procedures (4, 7, 9, 20, 21) has led some workers to believe that histamine is contained, in a diffusible form, within a cytoplasmic inclusion. To test this hypothesis, studies were undertaken using means which would alter the physical properties of a membrane. Described in this paper are the effects of various histamine liberators tested under such conditions.

[1]Manuscript received September 16, 1958.
Contribution from the Department of Physiology, McGill University, Montreal, Que. The work was supported by a grant from the Lasdon Foundation.

Methods

Experiments were carried out using dog liver particle suspensions and isolated perfused cat paws.

Preparation of the Dog Liver Particle Suspension

The preparation was similar to that used by other workers (8, 9). The liver was removed from mongrel dogs under sodium pentobarbital anesthesia, after the bile duct had been securely ligated. The gall bladder was dissected away and the liver was immediately perfused with ice-cold saline (0.9%) until the perfusate appeared blood-free. Three portions, approximately 50 g each, were minced and ground with sand in 10 to 15 ml of ice-cold isotonic sucrose solution (0.32 *M*). Sand and tissue fragments were removed by decanting and filtering through several layers of gauze. The mixture was washed twice with isotonic sucrose. Nuclei and cellular debris were removed by centrifuging at 600 *g* for 5 minutes. (All centrifugation in these experiments was carried out at 0° C.) A final suspension of the large granule fraction was made by centrifuging at 8500 *g* for 10 minutes, decanting and resuspending the granules in a volume of isotonic sucrose to give a concentration of 2 g/ml with regard to the original tissue. The suspension was either used at once or stored at −2° C. The histamine content of the final suspension ranged between 3 and 10 μg/ml, less than 10% being in the liquid phase. The suspension was fairly stable, the spontaneous release was 1 to 8% per day. However, it was noted that when preparations over one week old were incubated the histamine was released more quickly; for this reason no suspension over 4 days old was used.

Incubation of the Dog Liver Particle Suspension

One milliliter of suspension was added to four milliliters of sucrose or mannitol solution, buffered at pH 8 with Tris(hydroxymethyl)aminomethane, 0.07 *M*, containing appropriate amounts of the liberator to be tested. The mixture was incubated at 37° C for periods of 15 or 30 minutes. Control samples containing buffer and liver particles but no liberator were treated in the same manner. In the first experiments incubation was carried out in a water bath, the particulate matter being kept well suspended by frequent shaking of the samples. In later experiments a Dubnoff metabolic shaking incubator was used. Following incubation the samples were immediately centrifuged at 30,000 *g* for 5 minutes; the decanted supernates were placed in a boiling water bath for 2 minutes. The practical purpose of routinely boiling the samples was to eliminate, to a reasonable degree, the action of any histaminase in the samples. The samples were then stored at −2° C until they were assayed.

The total content of histamine in the suspension before incubation was determined by assaying a sample of the suspension which had been heated to boiling. Of this total, a small percentage was always found in the liquid phase. The quantity of this non-sedimentible (or free) histamine was determined by adding 1 ml of the suspension to 4 ml of buffer and immediately

centrifuging at 30,000 *g* for 5 minutes, the supernate was then boiled and saved for assay. The amount of histamine in the sample supernates after boiling, minus the free histamine, represents the histamine released, and is expressed as a percentage of the total bound histamine.

Preparation and Perfusion of the Cat Paw

The procedure used was a modification of the technique of Högberg *et al.* (11). Under chloralose anesthesia the forepaws were removed just proximal to the radiocarpal joint. The radial artery was cannulated with fine polyethylene tubing, and the paw immediately irrigated with 0.9% saline until the perfusate appeared blood free. It was then suspended, claws up, in an incubator at 37° C and perfused with fluid at the same temperature under sufficient pressure to give a venous outflow of approximately 0.5 ml/minute. The effluent dropped into a funnel and was collected in graduated tubes outside the incubator.

In each experiment one forepaw was perfused with isotonic sucrose or mannitol solution (0.32 *M*) and the opposite paw with a hypertonic solution (1.2 *M*) of the same solute. After the collection of three 5-minute control samples, the liberator, dissolved in 0.1 or 0.15 ml of saline, was injected into the cannula and the perfusion interrupted. The injection was made so that the point of the needle was close to the tip of the cannula; the dead space in the cannula was always less than 0.05 ml. Approximately 5 seconds were required after the injection of the liberator before the perfusion could be stopped. By using various dyes, the circulation time for these preparations was found to be approximately 15 seconds. From these figures one can calculate that, at the flow rate used in these experiments, the liberator was well into the vascular bed of the paw when the flow was halted. The flow was routinely interrupted for 45 seconds to allow the liberator to diffuse into the tissue spaces and make contact with the cells. Perfusion was resumed after this time and 5-minute samples collected over the next 25 minutes. In several experiments the flow was stopped during the control periods, this interruption of the perfusion caused no changes in the flow rate or the histamine content of the perfusate when the flow was resumed. The samples were placed in a boiling-water bath for 2 minutes and stored at 0° C until assayed.

Histamine Assays

The samples were assayed on the atropinized guinea pig ileum suspended in oxygenated Tyrode at 30° C. The composition of the Tyrode in g/l. was: NaCl—9, KCl—0.42, $CaCl_2$—0.24, $MgCl_2$—0.005, $NaHCO_3$—0.5, and glucose —1. When any compound tested altered the sensitivity of the ileum, equivalent amounts of it were added to the standard histamine solution. In the experiments with the liver homogenates the final concentration of the liberators was known and it could easily be determined whether or not the ileum would be affected by these concentrations. However, in the experiments with the cat paws the final concentration of the liberators in the venous

effluent was not known. The highest possible concentration could occur if all the liberator passed through the paw and appeared in the first sample. By calculating the maximum concentration that could have occurred in the first sample of each experiment, it was found that only saponin had any effect. This was a potentiating action which was so slight [22 nanograms (ng) histamine standard was equivalent to 20 ng histamine plus saponin] that it was ignored.

Mepyramine maleate reduced the responses of histamine and the samples to the same extent; this served as evidence that the substance assayed was histamine.

Other procedures used in the experiments are described in the text.

Results

The Release of Histamine from Dog Liver Particles Suspended in Isotonic and Hypertonic Sucrose Solutions

In a series of 31 experiments the histamine-releasing activity of six known histamine liberators was tested in both isotonic and hypertonic sucrose solutions (Fig. 1*a*). The rate of release in isotonic media was similar to that found by other workers (8). Release of histamine from particles suspended in 0.84 *M* sucrose solution, not shown in the graph, did not significantly differ from that released in the isotonic solution. However, when the particles were incubated in a 1.2 *M* sucrose solution, much less histamine was discharged by the base-type histamine liberators. As seen in Table I, the differences in the percentage of histamine released by the basic histamine liberators in isotonic and hypertonic sucrose solutions were highly significant.

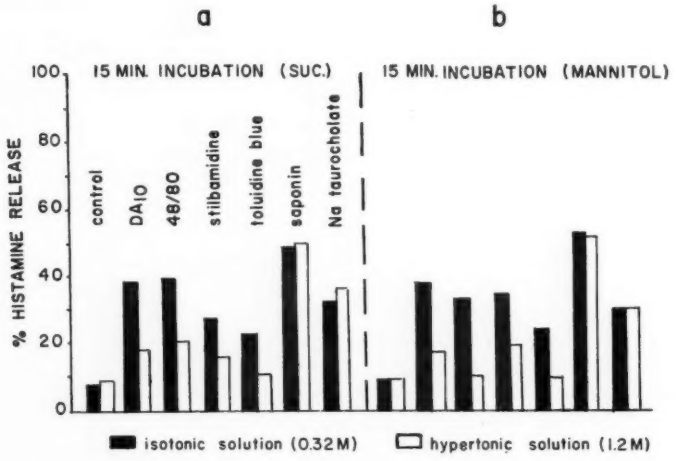


FIG. 1. Release of histamine from dog liver particles incubated at 37° C with histamine liberators in isotonic and hypertonic (1.2 *M*) solutions of *a*, sucrose and *b*, mannitol.

No significant differences could be found with the surface-active compounds saponin and sodium taurocholate or in the spontaneous release in the control samples.

Concentrations of the liberators were chosen to give rates of release which were of the same magnitude, although as can be seen from the standard error of the mean in Table I, there was a wide variation in the amount of histamine released by the same liberator from different suspensions. On the basis of molecular weight, toluidine blue and 48/80* were the most active liberators. Both were about twenty times as active as sodium taurocholate, which was the least effective of the compounds tested. With all of the basic histamine liberators used, the release of histamine in hypertonic sucrose solutions was approximately 50% less than that in the isotonic media.

TABLE I

Release of histamine from dog liver particle suspensions by various histamine liberators in isotonic (0.32 *M*) and hypertonic (1.2 *M*) sucrose solutions

Liberator	No. expts.	Conc., μg/ml	Incub. time, min	% Histamine released (mean ± S.E.M.) 0.32 *M*	1.2 *M*	*P*
Control	10	—	15	7±3	8±2	>.05
			30	15±5	13±3	>.05
DA_{10}	10	200	15	39±9	18±5	<.01
			30	67±9	29±7	<.01
Stilbamidine	4	200	15	27±3	16±2	<.01
			30	60±7	32±2	<.01
Toluidine blue	5	10	15	23±5	10±2	<.01
			30	54±4	23±5	<.01
48/80	5	60	15	40±13	22±4	<.01
			30	66±20	39±9	<.05
Saponin	5	400	15	49±13	50±13	>.05
			30	86±11	82±13	>.05
Sodium taurocholate	4	1000	15	32±5	36±5	>.05
			30	65±10	68±8	>.05

The rate of histamine release from the liver particles appears to be in a linear relationship with time for all the liberators except saponin, 48/80, and DA_{10} (1,10-diaminodecane), Table I. However, by plotting the results of individual experiments (Fig. 2), all the liberators, including these three, show this linear relationship. In other words, the release of histamine from the liver particles is a slow continual process. This is in contrast to the release in the cat paws, as will be shown later.

It was found that the effect of the 1.2 *M* sucrose solution on the release of histamine from these preparations was reversible, as it could be altered by changing the tonicity of the media. The evidence is as follows: six samples of liver particle suspension were incubated with DA_{10}; three in isotonic and

*A mixture of low polymers of *p*-methoxy-N-methylphenylethylamine, chiefly dimer, trimer, and tetramer. Kindly supplied by The Wellcome Research Laboratories, Tuckahoe, N.Y.

three in 1.2 *M* sucrose solutions. After 15 minutes' incubation, two samples, one of each tonicity, were removed, centrifuged at 30,000 *g* for 5 minutes, resuspended in isotonic sucrose containing the same concentration of DA_{10}, and incubated for another 15 minutes. The other four samples served as controls, two (one of each tonicity) were incubated for 15 minutes, and two for 30 minutes. The results of this experiment are shown in Fig. 3. When the sample suspended in isotonic sucrose solution was resuspended in the isotonic solution there was an increase of 32% in histamine release over its

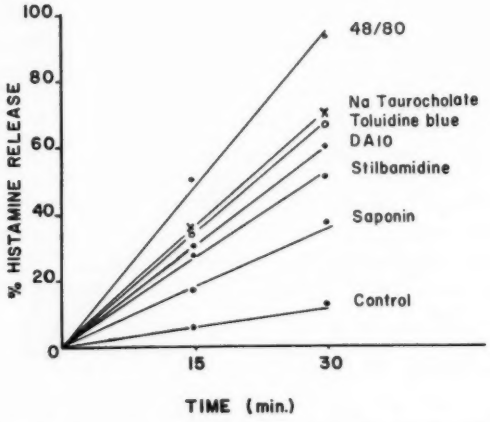


FIG. 2. Release of histamine from dog liver particles showing the linear relationship with time. The particles were incubated in isotonic sucrose at 37° C. Concentrations of the liberators are given in Table I. Each curve represents one experiment.

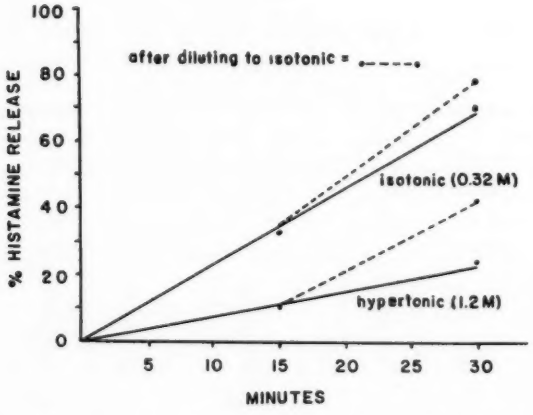


FIG. 3. Histamine release from dog liver particles incubated with DA_{10} (100 μg/ml) in isotonic and hypertonic sucrose. The broken lines show the release from particles resuspended in isotonic sucrose.

control. However, the increase in histamine release from the hypertonic sample which was resuspended in the isotonic solution was 182% greater than its corresponding control. Undoubtedly some of the increased histamine in the two treated samples was the result of mechanical damage to the particles incurred during the resuspension of them. Nevertheless, the results clearly indicate that the effect of the 1.2 *M* sucrose solutions on the particles is reversible.

The Release of Histamine from Dog Liver Particles Suspended in Isotonic and Hypertonic Mannitol Solutions

Table II summarizes the results of 22 experiments in which mannitol solutions were used as the suspending media. These results are much the same as those found with sucrose solutions (Fig. 1*b*). No significant differences were found between the percentages of histamine released in sucrose and mannitol solutions; it was therefore found convenient to group all of the results together in analyzing the difference between the percentage of histamine released in the isotonic and hypertonic solutions.

TABLE II

Release of histamine from dog liver particle suspensions by various histamine liberators in isotonic (0.32 *M*) and hypertonic (1.2 *M*) mannitol solutions

Liberator	No. expts.	Conc., μg/ml	Incub. time, min	% Histamine released (mean ± S.E.M.) 0.32 *M*	1.2 *M*	*P*
Control	4	—	15	10±2	10±2	>.05
			30	25±3	24±4	>.05
DA_{10}	4	200	15	39±9	18±5	<.01
			30	67±9	29±7	<.01
Stilbamidine	4	200	15	35±3	19±7	<.01
			30	66±4	41±13	<.01
48/80	3	60	15	35±10	13±7	<.01
			30	64±14	37±9	<.05
Toluidine blue	4	10	15	25±5	10±7	<.01
			30	60±12	29±12	<.01
Saponin	4	400	15	54±18	52±19	>.05
			30	75±16	73±14	>.05
Sodium taurocholate	4	1000	15	30±8	30±6	>.05
			30	64±14	66±10	>.05

The Release of Histamine from Isolated Cat Paws Perfused with Isotonic and Hypertonic Sucrose Solutions

Figure 4 shows the results of six experiments, three each with DA_{10} and 48/80, on the release of histamine from cat paws perfused with isotonic and hypertonic (1.2 *M*) sucrose solutions. Before samples were collected, each paw was perfused for 1 hour. The paw perfused with the 1.2 *M* solution became dehydrated as was evidenced by a weight loss of 3.5–5.0% between the 5th and 60th minutes of perfusion. No weight changes were noted in the

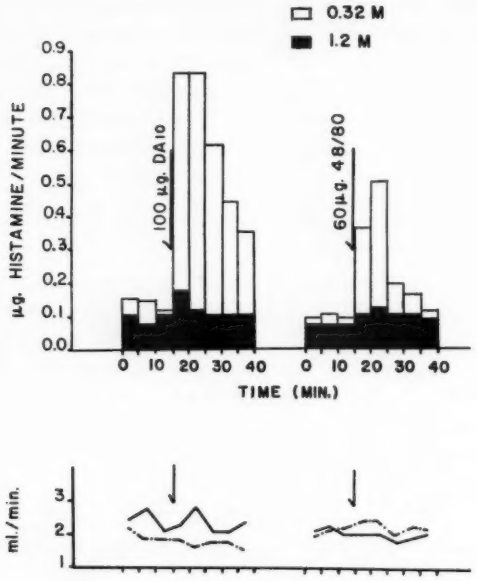


FIG. 4. The discharge of histamine from isolated cat paws perfused with isotonic and hypertonic sucrose solutions. Below are the flow rate curves. The solid lines represent the average venous outflow from paws perfused with 0.32 *M* sucrose. The broken lines indicate the flow from paws perfused with 1.2 *M* solutions. The arrows indicate the time of injection.

preliminary perfusion with the isotonic sucrose solution. After three 5-minute control samples were collected, 100 μg of DA_{10} or 60 μg of 48/80 were injected into the cannula.

In the paws perfused with isotonic sucrose solutions, DA_{10} caused an immediate increase in the histamine content of the venous effluent. The histamine content of the succeeding samples decreased slowly over the next 25 minutes, giving an average total release of 10 μg of histamine in excess of the spontaneous release calculated to occur in that period. The average total release of histamine from the paws perfused with the 1.2 *M* solution was 0.15 μg.

Similar results were obtained with 48/80, but the dose used was less effective than that of DA_{10}. An average of 6.0 μg of histamine was discharged from the paws perfused with the isotonic solution, while only 1.3 μg was cleared from the paws perfused with the hypertonic media. The small amount of histamine released by 48/80 in these preparations is in contrast to the extreme effectiveness of this drug found by Högberg *et al.* (11). In a similar experiment in which one paw was perfused with Tyrode and the opposite one with 0.32 *M* sucrose solution, approximately ten times as much histamine was

liberated from the paw perfused with Tyrode. Using Tyrode and isotonic sucrose solutions, Mongar and Schild (15) found roughly this same ratio in histamine release from the mitochondrial fraction of guinea pig lungs.

As can be seen from the flow rate curves in Fig. 4, there was no evident vasoconstriction after the injection of either DA_{10} or 48/80. In several experiments in which histamine itself was injected into the cannula, doses as high as 0.1 mg produced no decrease in the rate of venous outflow. When the paws were perfused with Tyrode, the typical vasoconstriction was seen after the injection of the liberators or histamine. The reason for this failure of exogenous or liberated histamine to produce constriction of the arterioles is not known; however, it enables the study of histamine release without the complications produced by vasoconstriction.

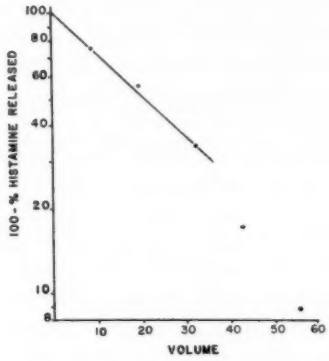


FIG. 5. Histamine output from perfused cat paws in one experiment, after the injection of 100 μg of DA_{10}. Ordinate: the total output of histamine was taken as 100%, the points show the percentages still remaining in the paw. Abscissa: the volume of perfusate expressed as a percentage of the paw's weight.

The course of histamine release from the perfused cat paws appears to be such that equal fractions of histamine remaining in the paw are released into equal volumes of perfusate. This type of release was first shown by Feldberg and Paton (5) using the perfused cat gastrocnemius. Figure 5 shows the results of a typical experiment in which histamine was released from the cat paw by DA_{10}. The abscissa is the volume of the perfusate expressed as a percentage of the paw's weight, assuming a fixed relationship between weight and volume. The findings suggest an explosive release of histamine into the extracellular space, which is then washed out by the perfusate. The dropping off of the last two points can best be explained by the formation of edema which would effectively decrease the concentration of histamine in the interstitial space and thereby decrease the amount washed out by the perfusion fluid.

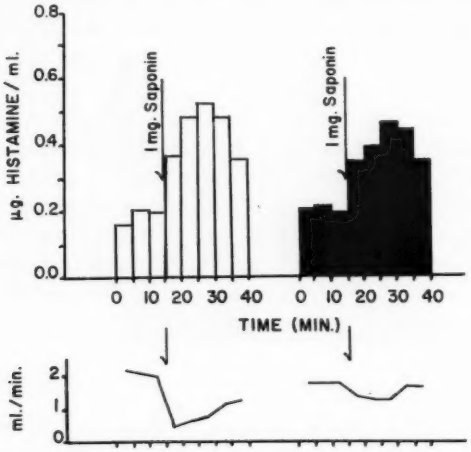


FIG. 6. Histamine liberation by saponin from isolated cat paws. The histogram on the left shows the release from paws perfused with 0.32 *M* sucrose solutions; the one on the right, that released from paws perfused with 1.2 *M* solutions. Below are the flow rate curves. The arrows indicate the time of injection. Each histogram is the average of three experiments.

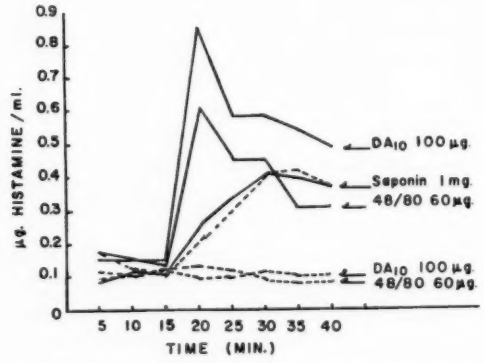


FIG. 7. Histamine release by DA_{10}, saponin, and 48/80 from cat paws perfused with mannitol solutions. The solid lines show the release in 0.32 *M* media and the broken lines that in 1.2 *M* solutions. Injections were made at the 15-minute mark.

Figure 6 shows the results of three experiments on the release of histamine from perfused cat paws by saponin. Unlike DA_{10} and 48/80, which produced no change in the pressure–flow relationship, the injection of 1 mg of saponin into the cannula increased the resistance to flow and resulted in the appearance of a bloody tint in the effluent. The reduction of flow might have been due to the clogging of small vessels by proteins or desquamated cellular material

rather than to vasoconstriction. The reduction in flow occurred in both solutions, though to a lesser extent in the hypertonic media. As might be expected from the slower flow rates, the concentration of histamine in the venous outflow rose more slowly and remained elevated longer. Again in these experiments there was no significant difference between the amount of histamine released in the isotonic and 1.2 *M* sucrose solutions. An average of 2.04 μg of histamine was released in the isotonic solution and an average of 1.98 g was discharged from the paws perfused with the hypertonic solution.

The Release of Histamine from Isolated Cat Paws Perfused with Isotonic and Hypertonic Mannitol Solutions

The results of nine experiments in which the isolated cat paws were perfused with 0.32 *M* and 1.2 *M* mannitol solutions are shown in Fig. 7. The results were much the same as those obtained with sucrose solutions. No decrease in the flow followed the injection of DA_{10} and 48/80, but a decrease did occur after the injection of saponin. In most cases there was no additional release of histamine above the control values after the injection of DA_{10} or 48/80 in the paws perfused with 1.2 *M* mannitol solutions.

Discussion

Mast cell granules have been reported by several workers to possess a limiting boundary (1, 13, 22). These observations along with those of Zollinger (24) that the granules exhibit an osmotic behavior, suggest that the mast cell granules are clearly defined structures possessing a surrounding membrane. The association of heparin (12) and histamine (20) with these granules leave two possibilities regarding their attachment to the granule: (*a*) they are situated on the membrane or (*b*) they are contained within the granule. It is more appealing to think of them as being within the granule in view of the fact that histamine can be released by forces which would tend to disrupt a membrane.

If histamine and heparin are inside the granule, the liberators must in some way induce a transfer of these compounds across the membrane. This could be accomplished in several ways: (*a*) the liberator could inhibit an active transport system which might be supposed normally to keep these substances confined within the granule—though theoretically possible, this seems highly improbable in view of the very slow rate of histamine release from suspensions stored for long periods at temperatures low enough to inhibit any transport mechanism; (*b*) they could also release histamine and heparin chemically bound in the granule thus allowing their free diffusion across the membrane; (*c*) they could release these active substances by altering the structure of membrane itself. A fourth possibility is that they could be attracted into the granule and by an osmotic loading effect cause a swelling of the granule. This would result in an increase in the permeability of the granule membrane allowing the substances to diffuse out. It is this last possibility that will be proposed here.

It can be shown that all the basic liberators used in these experiments will form a complex with heparin. This affinity of the liberator for heparin may well explain the specificity of their action, and suggests that they do not act directly on the membrane but rather unite with heparin, possibly as an ion exchange. The consequence of the ion exchange would be an increase in the number of osmotically active particles, resulting in an osmotic loading of the mast cell granule. Swelling would be expected to occur causing rupture of the cell and a freeing of the particles. Dougherty and Gottlieb (3) have observed swelling of the mast cell granules during the slow disintegration of mast cells in the rat subcutaneous tissue.

In what way does the 1.2 *M* sucrose solution interfere with the release of histamine? As far as is known, the only effect of sucrose, which is a relatively nonpenetrating molecule, is to set up an osmotic gradient across the membrane, causing a decrease in the volume of the structure. Whether this could cause a decrease in the permeability of the membrane cannot be said. However, if such is the case, the reduction in the histamine released in the 1.2 *M* solutions could be considered as the result of fewer liberator molecules entering the granule per unit time.

A second and more acceptable explanation for the effect of the 1.2 *M* sucrose solutions is that the osmotic gradient set up by the sucrose counteracts that set up in the granules by the entrance of liberator molecules. This would prevent the granules from swelling so easily, and thus the release of histamine would be retarded. The fact that the reduction in histamine release was approximately the same with all the basic liberators makes this supposition more likely. Comparable results obtained in experiments where mannitol was used instead of sucrose demonstrate that sucrose is not specifically responsible for the effects found in the 1.2 *M* solutions.

According to Rideal and Taylor (19), saponin and anionic detergents cause hemolysis of red blood cells by attacking the lipoprotein–cholesterol complex. This destruction is accompanied by a change in the cation permeability, a swelling of the cell, sphering, and a breakdown of the cell-wall complex. It is assumed that the same sequence occurs with other membranes. There is no reduction in the histamine released by these compounds in the 1.2 *M* solutions because the holes made by the lytic action probably allow the entrance of sucrose into the granule or cell thus annulling the osmotic gradient originally caused by the hypertonic solution. Saponin has no special affinity for the mast cells; this may be the reason for its weak action in releasing histamine from the perfused cat paws.

The results support the view that histamine is contained within a cytoplasmic granule and that, in so far as the basic histamine liberators are concerned, histamine release is due to an osmotic loading action on the particle.

Acknowledgments

The author wishes to express his thanks to Dr. H. Garcia-Arocha and Dr. F. C. MacIntosh for their aid in preparing this manuscript, and to Miss G. Dudas for technical assistance in assaying the samples.

References

1. Bloom, G., Larsson, B., and Smith, D. Acta Pathol. Microbiol. Scand. **39**, 1 (1957).
2. Coopenhaver, J. H., Jr., Nagler, M. E., and Goth, A. J. Pharmacol. Exptl. Therap. **109**, 401 (1953).
3. Dougherty, T. F. and Gottlieb, L. S. Federation Proc. **17**, 143 (1958).
4. Fawcett, D. J. Exptl. Med. **100**, 217 (1954).
5. Feldberg, W. and Paton, W. D. M. J. Physiol. **114**, 490 (1951).
6. Garcia-Arocha, H., Ashwin, J. G., and Grossberg, A. L. Abstr. XIX Intern. Physiol. Congr. 378 (1953).
7. Grossberg, A. L. Ph. D. thesis. McGill University. 1953.
8. Grossberg, A. L. and Garcia-Arocha, H. Science, **120**, 762 (1954).
9. Hagen, P. Brit. J. Pharmacol. **9**, 100 (1954).
10. Higginbotham, R. D. and Dougherty, T. F. Federation Proc. **16**, 252 (1957).
11. Högberg, B., Thufvesson, G., and Uvnäs, B. Acta Physiol. Scand. **38**, 135 (1956).
12. Jorpes, E., Holmgren, H., and Wilander, O. Z. mikroskop-anat. Forsch. **42**, 279 (1937).
13. Köksal, M. Acta Med. Turcica, **5**, 85 (1953).
14. Mongar, J. L. and Schild, H. O. J. Physiol. **126**, 44P (1954).
15. Mongar, J. L. and Schild, H. O. J. Physiol. **131**, 207 (1956).
16. Mongar, J. L. and Schild, H. O. J. Physiol. **135**, 301 (1957).
17. Mota, I., Beraldo, W. T., and Junqueira, L. C. U. Proc. Soc. Exptl. Biol. Med. **83**, 455 (1953).
18. Mota, I. Brit. J. Pharmacol. **12**, 453 (1957).
19. Rideal, E. and Taylor, F. H. Proc. Roy. Soc. B, **146**, 225 (1957).
20. Riley, J. F. and West, G. B. J. Physiol. **120**, 453 (1953).
21. Schild, H. O. Quart. J. Exptl. Physiol. **26**, 165 (1936).
22. Smith, D. E. and Lewis, Y. J. Biophys. Biochem. Cytol. **3**, 9 (1957).
23. Trethewie, E. R. Australian J. Exptl. Biol. Med. Sci. **16**, 22 (1938).
24. Zollinger, H. U. Experientia, **6**, 384 (1950).

THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE AND HUMIDITY ON SHOCK PRODUCED BY A CLAMPING PROCEDURE[1]

R. E. HAIST, REBEKA MOSCARELLO, T. L. FRIEDLICH, AND J. R. HAMILTON

Abstract

The influence of environmental temperature on the development of shock produced by a clamping technique in rats was studied. In experiments in which the animals were subjected to different environmental temperatures during the period of limb ischemia, the best survival was obtained with an air temperature of 15° C. At 9.5° C and 40° C the rats did not survive the 10-hour clamping period. When the clamping was carried out at a standard temperature (27° C) and the rats were then transferred to a room at different temperatures just prior to clamp release, the best survival was obtained at or near a temperature of 24° C. The temperature in the colon of the shocked rats fell quickly in a cooler environment and rose in a warmer one. When chlorpromazine (0.35 mg/100 g rat) was given at the time of clamp removal to rats kept thereafter at 9.4° C, 20–21° C, 24° C, and 30° C, survival was significantly prolonged at air temperatures of 20–21° C, but not at 9.4° C, 24° C, or 30° C. Changes in humidity had no significant influence on survival. The experiments show that the optimum temperature during the period of ischemia is different from that for hindering the development of shock following a period of ischemia.

Environmental change may influence the response of an animal to physical injury by altering its temperature, or its loss of water, or by causing circulatory changes. The temperature of an animal with small mass rather quickly reflects the environmental temperature but this may not be true of larger animals. If the temperature of a tissue is lowered, however, then certain chemical reactions within the tissue will go on more slowly, and if, at this time, a tissue has its blood supply seriously reduced, then the deleterious effects of the ischemia will take longer to appear. Reduction in body temperature prolongs the tolerated period for circulatory arrest (1) and increases the duration of ischemia that can be withstood by liver (2, 3), kidney (4, 5), brain (6, 7), and rats' tails (8). The breakdown of high energy phosphorus compounds in ischemic muscle is slowed down (9) and the period of low blood pressure that can be sustained without developing irreversible shock is lengthened (10, 11). It should be noted that a diminishing tolerance to a standard haemorrhage has been found when the body temperature falls *below* 31° C (12). Various workers have studied the optimal temperatures for survival in shock. These vary considerably. It seems reasonable that temperature changes may have different effects if the change occurs during a period when tissues are receiving an insufficient blood supply or if it takes place after a period of ischemia has occurred. The effects of environmental change during the ischemic period and following an ischemic period can be studied in animals in which the circulation to the limbs is cut off by tourniquets or clamps.

[1]Manuscript received August 29, 1958.
Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

Chlorpromazine alone and with reduced body temperature has been reported to have a beneficial effect in shock secondary to haemorrhage (11, 26) though others found that when this drug was administered at a considerable time after the haemorrhage, no significant effect on survival was observed (13, 14). As we shall see from the present study, temperature does influence this effect. There is little or no information concerning the influence of environmental humidity on the response of an animal to injury, though this may influence fluid loss, and some calculations of the effect of fluid loss at different environmental temperatures have been made (15).

Materials and Methods

Female rats of the Wistar strain were suspended in adhesive-tape slings and shocked by a clamping technique (16). This involved the cutting off of the circulation to both hind legs for a period of 10 hours, following which the clamps were removed and the circulation to the extremities re-established. Survival times were measured by direct observation and by recording the heart beats (17). A standard environmental temperature of 27° C was obtained by using a constant-temperature box. Different environmental temperatures and humidities were obtained by the use of a room in which temperature and humidity could be altered. This room did not function perfectly but the actual temperatures and humidities were recorded. Body temperatures were measured by means of thermocouples introduced by way of the rectum and inserted to a depth of 50 mm. Chlorpromazine (0.35 mg/100 g body weight) was given by subcutaneous injection to some of the rats just prior to release of the clamps.

Experimental Results

Four experiments were undertaken. In the first experiment, a study was made of the effect of different environmental temperatures during the period of limb ischemia. In this experiment the rats were placed in the room at the given temperature during the period when the clamps were in place. The rats were then transferred to the constant-temperature box at 27° C at the time of clamp removal. The humidity ranged from 10–44% R.H. during this experiment. The survival times are given in Table I. The colonic temperatures are shown in Fig. 1. From the table it will be evident that the best survival occurred when the clamping was carried out at an environmental temperature of 15° C. At 9.5° C and at 40° C the rats did not survive the 10-hour clamping period. This may be, in part, a result of their being relatively immobilized in the slings. The colonic temperatures of Fig. 1 show that these rats are unable to maintain their body temperature in a cold environment when movement is restricted. At an environmental temperature of 15° C the body temperature fell to 24° C in the clamped animals.

These results are in keeping with those of Allen (18) which showed that local asphyxia in a ligated limb had a more rapid effect at higher local temperature than at low.

TABLE I

Survival times in rats maintained at the indicated environmental temperatures during the period when the clamps were in place
(After clamp release all animals were at 27° C.)

Environmental temperature, °C	Relative humidity, %	Number of rats	Mean weight, g	Mean survival time, hours
9.5	44	12	219	0
15	26	12	206	10.3
20	20	12	200	6.2
27	—	21	204	4.4
34	10	12	208	6.3
40	10	10	198	0

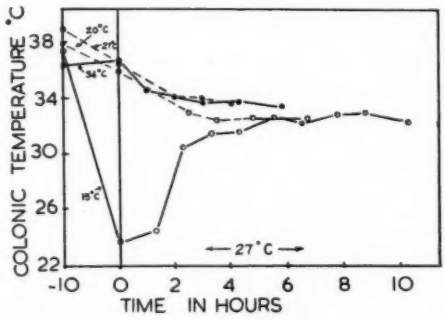


FIG. 1. Body temperatures in rats maintained at the indicated environmental temperatures during the period when the clamps were in place. At zero time the clamps were removed. After clamp release, all animals were kept at 27° C.

In the second experiment, the clamping was carried out at a standard temperature of 27° C in a constant-temperature box and, just prior to clamp release, the rats were transferred to the room at the different temperatures. This permitted observations to be made on the effects of environmental temperature on events following a standard period of limb ischemia. The survival times are given in Table II and the colonic temperature changes in Fig. 2. From the results it appears that the best survival times were obtained at environmental temperatures of 24°–25.5° C. It is seen too from the colonic temperature chart that after clamp removal, the body temperature falls quickly in a cooler environment and rises in a warmer one. When the environmental temperature was below 30° C the colonic temperature fell in these shocked rats. At an environmental temperature of 40° C the body temperature rose. At an environmental temperature of 24–25° C the body temperature fell to 30–31° C. At room temperatures then, the rats became hypothermic. Because of infection in the rat colony and mechanical breakdown in the control of the constant-temperature room, the experiment could not be carried further at this time. Later, with a new supply of rats,

TABLE II

Survival times in rats maintained at 27° C during the period when the clamps were in place (After clamp release the animals were kept at the temperatures indicated.)

Environmental temperature, °C	Environmental humidity, %	Number of rats	Mean weight, g	Mean survival time, hours
9.5	44	12	209	3.9
15	26	12	202	5.1
20–21.5	20–25	29	209	5.7
24–25.5	25–28	28	208	9.2
27	—	21	216	4.5
31	24	7	212	2.3
34	10	12	201	2.1
40	10	8	194	0.8

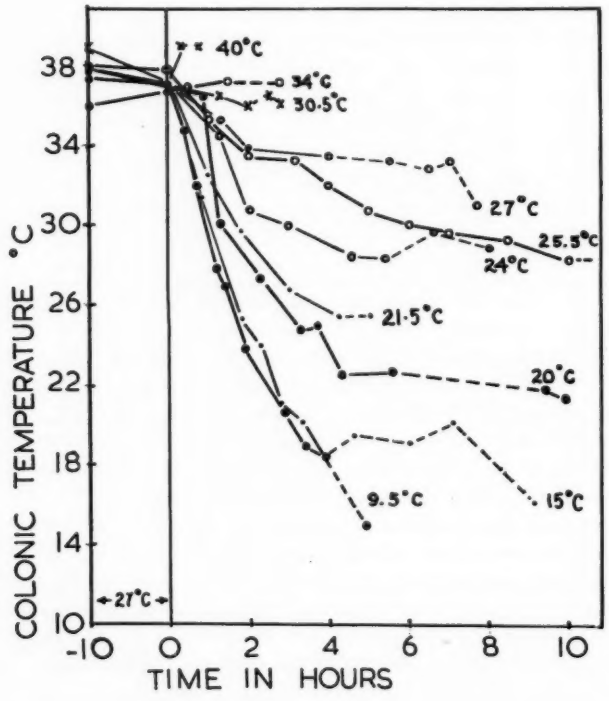


FIG. 2. Body temperatures in rats maintained at 27° C during the period when the clamps were in place. At zero time the clamps were released. After clamp release the animals were kept at the temperatures indicated. The solid line ends at the point where 50% of the rats were dead.

from a different source (Carworth Farms), a third experiment was carried out along much the same lines as experiment 2, but to which were added observations on the effects of chlorpromazine (0.35 mg/100 g rat), at three different temperature levels. Other rats were placed in slings but not clamped in order to see what effects the restriction of activity might have per se. The survival times are given in Table III and Fig. 3 and the colonic temperature changes are shown in Figs. 4 and 5. These show that even animals suspended in slings, but not clamped, exhibit some reduction in body temperature in the cold, though the fall is not as rapid and extensive as in the shocked animals. The apparatus used for recording body temperatures in this experiment recorded no lower than 16° C and temperatures below this were recorded as 16° C. When such values are included in the mean figures on the charts, the lines are dotted.

The results indicate that a temperature of 24° C is optimal for survival. However, as will be seen from Table III, the difference between the survival times at 24° C and 27° C was not statistically significant ($P > .05$) and between 24° C and 20° C was only probably significant ($P = .05$). The survival times at 24° C were significantly greater than those at 9.4° C, 16° C, 30° C,

TABLE III

Survival times in rats maintained at 27° C during the period when the clamps were in place (After clamp release the animals were kept at the temperatures and humidities indicated. Mean values are followed by standard deviations. The rats receiving chlorpromazine received 0.35 mg per 100 g body weight.)

Environmental		Number of rats		Mean weight, g		Mean survival time, hours	
Temperature, ° C	Humidity, %	Untreated	Chlorpromazine-treated	Untreated	Chlorpromazine-treated	Untreated	Chlorpromazine-treated
10	82	10		217±10		3.8±1.0	
20	76	10		231±14		7.6±2.0	
27	75	8		229±14		7.8±2.7	
30	71	10		237±10		3.6±1.1	
9.4	41	10	10	213± 9	223±12	4.3±0.7	4.2±0.6
16	35	10		226± 4		6.6±0.8	
20–21	12–20	10	10	220± 7	227±17	7.1±2.3	10.3±2.0
21	12		10		222±12		9.3±2.0*
24	21	10		224±13		9.6±3.2	
27	42	10		246±16		7.5±3.3	
30	21	10	10	215±11	208± 8	5.4±3.0	6.2±2.6
34	19	9		235±22		2.3±0.5	
10	38	15		224±16		Unclamped controls	

*Chlorpromazine given 1.5 hours before clamp release.

NOTE: *t* test, survival times at low humidities (R.H. 12–42%):
between 24° C and 9.4° C, $P < 0.001$ (highly significant);
between 24° C and 16° C, $P < 0.01$ (significant);
between 24° C and 21° C, $P = 0.05$ (borderline);
between 24° C and 27° C, $P > 0.05$ (not significant);
between 24° C and 30° C, $P < 0.01$ (significant);
between 24° C and 34° C, $P < 0.001$ (highly significant);
between 9.4° C and 30° C, $P > 0.05$ (not significant); between 9.4° C and 21° C, $P < 0.01$ (significant);
between 21° C and 30° C, $P > 0.05$ (not significant).
t tests between survival times at low humidities (R.H. 12–42%) and high humidities (R.H. 71–82%) at 10° C, 20° C, 27° C, and 30° C, $P > 0.05$ (not significant).
t test between survival times of chlorpromazine-treated rats and controls at 20–21° C and R.H. 12–20% gave $P < 0.01$ (significant).

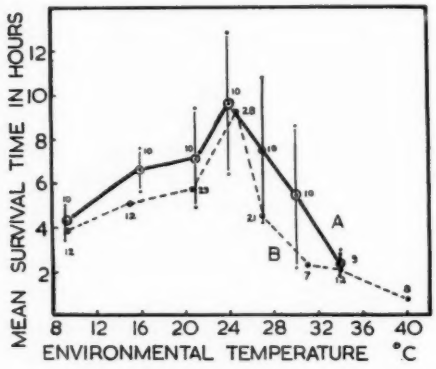


FIG. 3. Mean survival times in experiment 3 (solid line) and experiment 2 (dotted line) compared. The vertical lines indicate standard deviations in experiment 3. The figures show the number of rats per point.

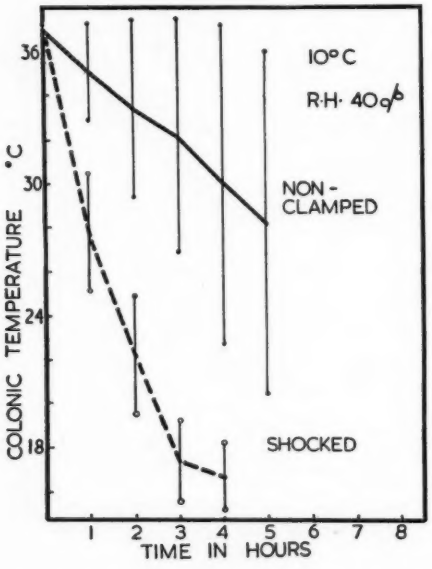


FIG. 4. Body temperatures in rats kept in slings but not clamped, at an air temperature of 10° C, compared to body temperatures in shocked rats, following clamp removal, under the same conditions. The vertical lines indicate standard deviations.

and 34° C. The difference in survival times between rats maintained at 9.4° C and 30° C was not significant. The survival time at 20–21° C was significantly longer than at 9.4° C but not significantly longer than the survival time at 30° C. When animals were kept at 9.4° C and 30° C the administration of chlorpromazine did not seem to have any significant effect on survival when given at the time of clamp release, and the changes in colonic temperature were similar for animals with and without chlorpromazine. However, at 20–21° C the administration of chlorpromazine did significantly prolong survival ($P > .01$) and the fall in colonic temperature was slightly less. In a further test at 24° C chlorpromazine did not have a statistically significant effect on survival. The changes in mean colonic temperature for these animals are shown in Fig. 6 along with the survival times.

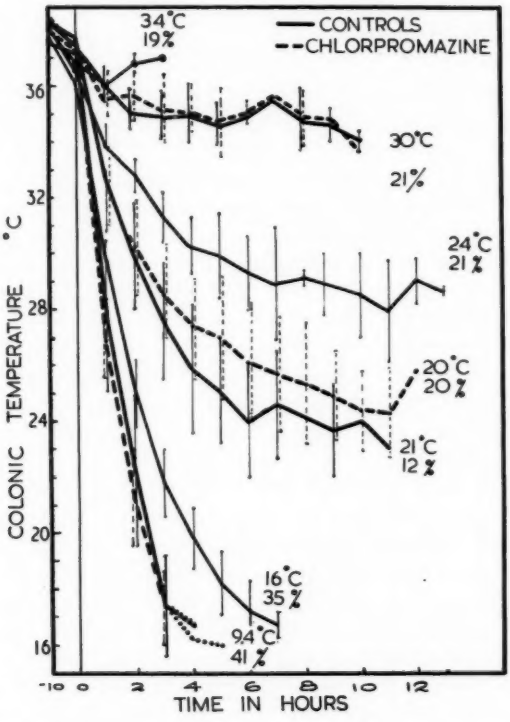


FIG. 5. Body temperatures in shocked rats with and without chlorpromazine (0.35 mg per 100 g body weight) at the air temperatures and humidities indicated. The relative humidities are given in per cent. Clamps were removed at zero time. Temperatures in shocked rats not receiving chlorpromazine are shown by the solid lines. Temperatures in shocked rats receiving chlorpromazine are shown by the discontinuous lines. The vertical lines indicate standard deviations. The fine dots indicate that the mean values shown are higher than the true values because of the limitations of the recording device.

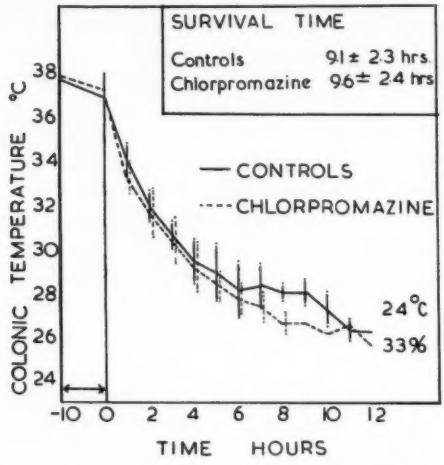


FIG. 6 Body temperatures in shocked rats with (dotted line) and without (solid line) chlorpromazine, at an environmental temperature of 24° C and relative humidity of 33%. Clamps were removed at zero time. Vertical lines indicate standard deviations. Mean survival times are followed by standard deviations.

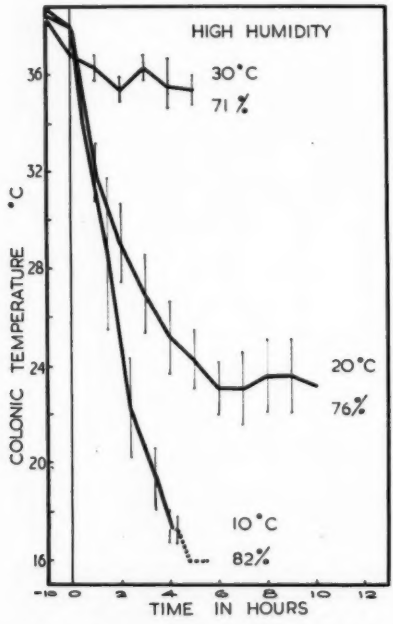


FIG. 7. Body temperatures in shocked rats at the air temperatures and humidities indicated. Relative humidities are given in per cent. The clamps were removed at zero time. Vertical lines show standard deviations. Where line is dotted it indicates that the mean figure shown is higher than the true value because of the limitations of the recording device.

The previous experiments were all carried out at relatively low humidities. A fourth experiment was performed, at three of the temperature levels, in which the relative humidity was maintained at or near 80%. The rat supply was the same as in experiment 3. The survival times are included in Table III. The colonic temperatures are shown in Fig. 7. The results do not indicate any great influence of rather large changes in environmental humidity on survival times in shock secondary to injury at environmental temperatures of 9–10° C, 20–21° C, and 30° C. The mean survival time at 30° C was shorter at the high humidity but this difference was not statistically significant. The colonic temperature changes were similar at the high and low humidities also.

Discussion

The results of these experiments indicate that the optimal temperature during the period of ischemia is different from that for the time following a standard period of ischemia i.e. when shock is developing. The best temperature for this latter period was about 24–25° C. This is in general accord with other reports which indicate an optimal temperature of 24° C for burn shock in rats (19), of 20° C for intestinal trauma in rats (20, 21), of 18–22° C for burn shock in mice (22). In nucleotide shock in mice, with potentially lethal doses of ATP, survival times were best at an air temperature of 25° C while in ischemic shock in mice the optimal temperature for recovery was reported to be closer to 18° C (23). Other workers indicated that a temperature of 25°–29° C was best for tourniquet shock in mice when adequate treatment with saline was given (24). Others concluded that the best temperature for survival was in the neighbourhood of normal body temperature (25) but Cleghorn indicated that the best survival after "critical" haemorrhage in dogs was obtained at temperatures of 70° F (15). The effect of hypothermia and chlorpromazine on shock following haemorrhage in dogs was investigated by Jaulmes, Laborit, and Benitte (26), Overton and DeBakey (11), and others (27, 28). The former lowered the body temperature in their animals to 28°–30° C while Overton and DeBakey maintained body temperatures around 31° C in their dogs. This degree of cooling of the body in itself prolonged survival, and chlorpromazine enhanced this effect. It is interesting that in our experiments an environmental temperature of 24–25° C in the postischemic period led to a fall in body temperature to 29–31° C. This temperature was the one at which the best survival times were obtained. In the rat then, hypothermia is achieved by leaving the animal at room temperatures, and hypothermia of this degree seems to prolong survival. When chlorpromazine was given at the time of clamp release at temperatures of 10° C, 24° C, and 30° C there was no significant effect on survival but at 20–21° C its administration did significantly prolong survival. The survival times were similar at 20–21° C regardless of whether the chlorpromazine was given 1.5 hours prior to, or at the time of, clamp release.

Relatively large changes in humidity caused no significant change in survival times, within the temperature range studied.

These various experiments point out the fact that the optimum temperature during the period when circulation to tissues is restricted or stopped will be different from the optimum temperature for hindering the development of shock following a standard period of such ischemia. It is important therefore to keep this distinction in mind when employing changes in body temperature in therapy. They show too that environmental temperature and body temperature are important in relation to the beneficial effect of chlorpromazine on survival.

Acknowledgment

This work was supported by a grant from the Defence Research Board of Canada.

References

1. Bigelow, W. G., Callaghan, J. C., and Hopps, J. A. Ann. Surg. **132**, 531 (1950).
2. Shumway, N. E. and Lewis, F. J. The physiology of induced hypothermia. Natl. Acad. Sci.–Natl. Research Council, Publ. No. **451**, 221 (1956).
3. Bernhard, W. F., Cahill, G. F., and Curtis, G. W. Ann. Surg. **145**, 289 (1957).
4. Moyer, J. H., Morris, G. C., and DeBakey, M. E. The physiology of induced hypothermia. Natl. Acad. Sci.–Natl. Research Council, Publ. No. **451**, 199 (1956).
5. Moyer, J. H., Heider, C., Morris, G. C., and Handly, C. Ann. Surg. **146**, 152 (1957).
6. Lougheed, W. M. and Kahn, D. S. J. Neurosurg. **12**, 226 (1955).
7. Pontius, R. G. and DeBakey, M. E. The physiology of induced hypothermia. Natl. Acad. Sci.–Natl. Research Council, Publ. No. **451**, 264 (1956).
8. Brooks, B. and Duncan, G. W. Ann. Surg. **112**, 130 (1940).
9. Threlfall, C. J. and Stoner, H. B. Brit. J. Exptl. Pathol. **38**, 339 (1957).
10. Antos, R. J. Proc. Soc. Exptl. Biol. Med. **56**, 60 (1944).
11. Overton, R. C. and DeBakey, M. E. Ann. Surg. **143**, 439 (1956).
12. Wilson, J. N., Marshall, S. B., Beresford, V., Montgomery, V., Jenkins, D., and Swan, H. Ann. Surg. **144**, 696 (1956).
13. Carruthers, G. F. and Gowdey, C. W. Can. J. Biochem. and Physiol. **34**, 217 (1956).
14. Gowdey, C. W., Kilborn, R. M., and Stevenson, J. A. F. Can. J. Biochem. and Physiol. **35**, 1241 (1957).
15. Cleghorn, R. A. Can. Med. Assoc. J. **49**, 355 (1943).
16. Haist, R. E. and Hamilton, J. I. J. Physiol. **102**, 471 (1944).
17. Smith, L. C. and Haist, R. E. Can. J. Biochem. and Physiol. **33**, 553 (1955).
18. Allen, F. M. Am. J. Surg. **45**, 459 (1939).
19. Elman, R., Cox, W. M., Lischer, C. E., and Mueller, A. J. Proc. Soc. Exptl. Biol. Med. **51**, 350 (1942).
20. D'Amour, F. E., Erickson, B. R., and Smith, D. L. Federation Proc. **14**, 33 (1955).
21. Erickson, B. R., Smith, D. L., and D'Amour, F. E. Proc. Soc. Exptl. Biol. Med. **94**, 1 (1957).
22. Bergman, H. C. and Prinzmetal, M. Arch. Surg. **50**, 201 (1945).
23. Green, H. N. and Stoner, H. B. Biological actions of the adenine nucleotides. H. K. Lewis & Co., London. 1950.
24. Tabor, H. and Rosenthal, S. M. Am. J. Physiol. **149**, 449 (1947).
25. Wakim, K. G. and Gatch, W. D. J. Am. Med. Assoc. **121**, 903 (1943).
26. Jaulmes, C., Laborit, H., and Benitte, A. Compt. rend. **234**, 372 (1952).
27. Fournel, J. Compt. rend. soc. biol. **146**, 561 (1952).
28. Courvoisier, S., Fournel, J., Ducrot, R., Kolaky, M., and Koetschet, P. Arch. intern. pharmacodynamie, **92**, 305 (1953).

TURNOVER OF PLASMA GLUCOSE IN ANESTHETIZED WARM- AND COLD-ACCLIMATED RATS EXPOSED TO COLD[1]

FLORENT DEPOCAS

Abstract

The turnover of plasma glucose has been measured in anesthetized warm- and cold-acclimated white rats by a method involving a single injection of a tracer amount of uniformly labelled C^{14}-glucose at 30° C, and measurement of the rate of fall of the plasma glucose specific activity in animals maintained at 30° C or moved to an environment at 6° C. Analysis of the data indicated a significant increase in the rate of disappearance of the labelled glucose when the warm-acclimated rats were transferred from 30° to 6° C but no significant change when the cold-acclimated rats were similarly treated. The concentration of plasma glucose remained essentially constant throughout the observation period. These results may be taken to indicate that the cold-induced increase in metabolism of anesthetized white rats is associated with an increase in plasma glucose turnover in warm-acclimated animals only.

Introduction

The demonstration of the ability of anesthetized warm- and cold-acclimated rats to give a metabolic response on exposure to cold (9) has provided a means of study of physiological events such as shivering (9) associated with the increased heat production of the cold-exposed animals and the role of the abdominal viscera (4) in this response. Anesthetized rats have now been used to investigate possible intermediary metabolic changes associated with temperature of acclimation or with the increased oxygen consumption of the cold-exposed anesthetized rat. This paper reports a comparison of plasma glucose turnover in warm- and cold-acclimated rats at 30° C (constant O_2 consumption) and during exposure to 6° C (increasing O_2 consumption).

Methods

Twelve male Sprague–Dawley rats, weighing on the average 218 g (range 198 to 246 g), were randomly assigned to two groups maintained for 5 to 10 weeks at 30° C (six rats) and 6° C (six rats). They were kept in separate cages, and Master Fox chow and tap water were supplied ad libitum. After acclimation the rats were anesthetized with sodium barbital (290 mg per kg, intraperitoneal) (9). One hour after anesthesia they were taped to a board in the supine position in a room at 30° C, the trachea was cannulated, and the right jugular and femoral veins were exposed. Twenty minutes after tracheal cannulation, 0.45 ml of a solution of 0.37 mg D-glucose[2] uniformly labelled with C^{14} (U-C^{14}-glucose) containing 39.1×10^6 counts/min was injected in the femoral vein. Blood samples (0.25 ml, 7 to 8) were then taken at intervals of 15 minutes from the same jugular vein in each rat and

[1]Manuscript received September 3, 1958.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 4995.
[2]Obtained from Atomic Energy of Canada Ltd., Commercial Products Division, Ottawa, Canada.

transferred to tubes containing 0.02 ml of heparin solution (10 mg per ml). The blood was kept at 6° C until further treatment. Thirty-two minutes after injection of the U-C^{14}-glucose, half the animals in each acclimation group were transferred to a room at 6° C while the others were maintained at 30° C. Oxygen consumption was calculated from measurements of oxygen partial pressure and volume of the CO_2-free exhaled gases (5).

Isolation of Plasma Glucose

Separation of plasma glucose has previously been made by paper chromatography of deproteinized–desalted plasma (15) and of deproteinized plasma (8). Both these procedures involve tedious details of technique. It was found that glucose would separate from the whole plasma, streaked directly on the paper, with *n*-propanol – ethyl acetate – water (7:1:2) as the chromatographic solvent. The following technique was then adhered to in all subsequent work. A 0.1-ml aliquot of the plasma was streaked across the top center of a $7\frac{1}{2}$-in. wide sheet of Whatman No. 1 paper. The streak was then flanked by two 5-microliter spots of plasma and the sheet dried for 1 hour in a well-ventilated fume hood. The paper was developed overnight by descending chromatography and dried at room temperature. The glucose was located by spraying with aniline phthalate the two lateral 1-in. strips cut from the chromatogram and heating the strips 10 minutes at 100° C. According to the position of the markers, a 5-cm wide strip, which had previously been shown to hold all the glucose in the unknown, was cut across the center portion of the chromatogram. The glucose was eluted with approximately 0.8 ml of water and the eluate collected in a 1-ml volumetric flask and made up to 1 ml. Aliquots of the eluate were taken for glucose determination by the Nelson method (13) and for C^{14} determination. Blank determinations of reducing substances on 13 strips of virgin paper carried through the chromatographic and elution procedures gave the equivalent of 8.0 ± 0.2 mg glucose per cent.

Two series of eight determinations of glucose on the same sample of plasma both by the chromatographic procedure described above (corrected for the blank) and direct analysis on the $Ba(OH)_2$–$ZnSO_4$ filtrate gave respectively 167.9 ± 1.8 and 182.6 ± 0.5 mg% glucose. The observed difference of 14.7 mg % between the two averages is most likely due in great part to the higher specificity of the chromatographic method (8). Six to seven replicate analyses on each of three other plasma samples gave an estimated standard error of ± 2.9 mg% for a single determination of glucose by the chromatographic procedure. The absolute recovery of glucose by this method was not determined as all plasma samples were treated similarly to obtain specific activities of plasma glucose.

C^{14} *Measurements*

The aqueous solutions of isolated glucose of assumed negligible solid contents were spotted on aluminum planchets and counted with a Nuclear-Chicago Model D47 Geiger counter with Micromil window and Model 166 scaling unit. The results were expressed as per cent injected C^{14} found per mg glucose.

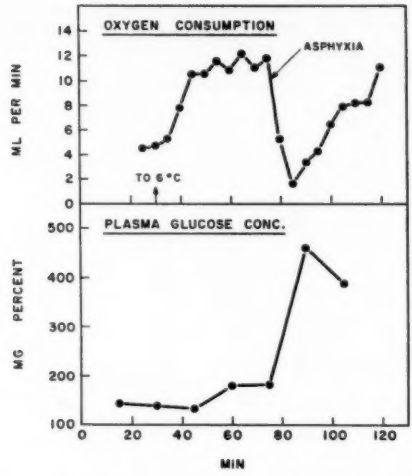


FIG. 1. Oxygen consumption and concentration of plasma glucose of an anesthetized 6° C acclimated rat transferred from 30° C to 6° C and experiencing respiratory difficulties due to temporarily obstructed tracheal cannula. The animal responded to asphyxia by a sharp increase in concentration of plasma glucose.

Results

Figure 1 illustrates the capability of the barbital-anesthetized rats used in this study to respond to asphyxia by a sharp rise in concentration of plasma glucose.

The average concentration of plasma glucose, the corresponding average specific activity, and the average oxygen consumption observed in the four groups of rats are given in Fig. 2. Rats kept at 30° C maintained nearly constant oxygen consumption while all cold-exposed rats reacted to the change in environment by an increase in oxygen consumption following a pattern described previously (1, 2). The average concentration of plasma glucose observed for all four groups of rats was lower at the second than at any of the other samplings for no obvious reason. Results for the same individuals at different sampling times did not otherwise differ significantly.

The specific activity of plasma glucose did not decrease linearly with time in the rats maintained at 30° C nor in the 6° C rats transferred to 6° C. A non-linear decrease with time in log specific activity of plasma glucose in normal rats given a single injection of C^{14}-labelled glucose has previously been observed by Baker *et al.* (2), who suggested its analytic description by the sum of two exponential functions corresponding to dilution of the labelled glucose into two different pools in the animals. However, the results obtained here on the 30° and 6° C rats maintained at 30° C could not satisfactorily be represented in this way as the successive least squares estimates of the four parameters specifying such a system, obtained from iterative approximations, did not converge to stable values.

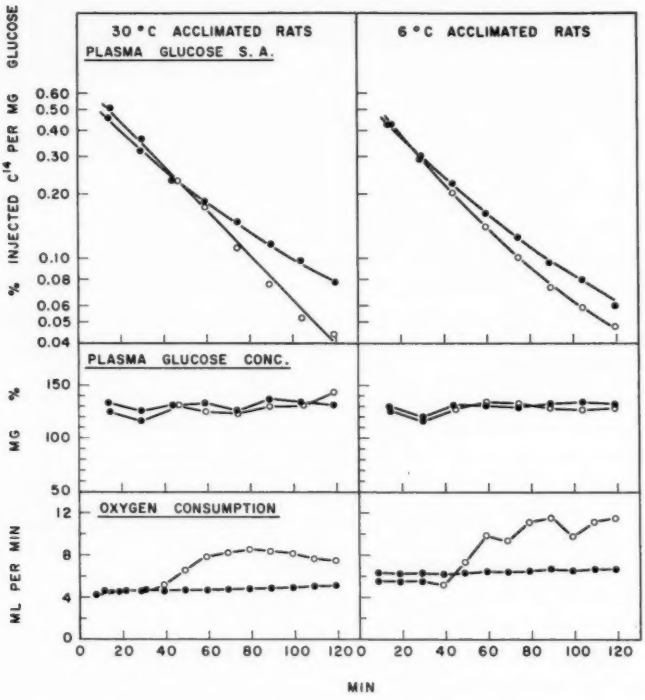


FIG. 2. Average oxygen consumption, concentration, and specific activity (S.A.) of plasma glucose of anesthetized 30° and 6° C acclimated rats observed after a single intravenous injection of U-C^{14}-glucose at 30° C (●) and after transfer to a cold room at 6° C (○).

In the 30° and in the 6° C rats transferred to 6° C the specific activity of plasma glucose apparently decreased at a faster rate at 6° C than in the 30° or 6° C rats maintained at 30° C. To test for significancy of this difference, the coefficients of the least squares parabolic regression of log specific activity of plasma glucose on time in minutes after injection of the U-C^{14}-glucose (log S.A. $= a+bt+ct^2$) were determined for each rat (Table I). For the 30° C rats transferred to 6° C (30 to 6) this regression was sensibly linear but for all other treatment groups the parabolic term was significant, the rate of decrease of log specific activity diminishing with time. From these equations, the mean rate of decrease of log specific activity of plasma glucose over the time intervals 0 to 32 minutes and 32 to 120 minutes was computed for each rat. Differences between group averages of these rates were then tested for significance (Table II). There was some variation in the results for individual rats of the same group, and the only treatment effect statistically demonstrable was an increase in the rate of decrease of log specific activity of plasma glucose on exposure of the 30° C acclimated rats to the cold environment.

TABLE I

Coefficients of parabolic regression of log glucose specific activity on time after injection of U-C^{14}-glucose into 30° and 6° C rats maintained at 30° C (30 to 30) or transferred to 6° C (30 to 6)

Treatment group	Rat No.	Coefficient		
		a	$-10^3\ b$	$10^5\ c$
30° C rats				
30 to 30	347	1.805	1.077	2.046
	334	1.752	1.037	2.558
	388	1.835	0.958	2.298
30 to 6	351	1.887	0.997	—
	344	1.767	1.004	—
	339	1.928	1.154	—
6° C rats				
30 to 30	355	1.725	1.196	2.506
	332	1.764	1.010	2.014
	330	1.875	1.053	2.030
30 to 6	354	1.973	1.543	4.214
	348	1.757	1.137	2.289
	333	1.783	1.335	2.402

TABLE II

Mean rates of decrease of log specific activity of plasma glucose after injection of U-C^{14}-glucose into 30° and 6° C rats maintained at 30° C (30 to 30) or transferred to 6° C (30 to 6)

Treatment group	Mean rate of decrease of log S.A.$\times 10^3$	
	15 to 32 min	32 to 120 min
30° C rats		
30 to 30 (1)	-9.16 ± 0.38	-6.74 ± 0.48
30 to 6 (2)	-10.51 ± 0.51	-10.51 ± 0.52
6° C rats		
30 to 30 (3)	-9.84 ± 0.49	-7.55 ± 0.33
30 to 6 (4)	-11.99 ± 0.92	-8.87 ± 0.53
Treatment difference		
(1)−(2)	1.35 ± 0.64	$3.77^{**} \pm 0.70$
(3)−(4)	2.15 ± 1.04	1.32 ± 0.62
½[(1)+(2)−(3)−(4)]	1.08 ± 0.61	-0.42 ± 0.47
[(1)−(2)]−[(3)−(4)]	-0.80 ± 0.85	$2.45^{**} \pm 0.66$

**Statistically significant to the 1% level.

Discussion

Little is known about the biochemical events associated with the immediate metabolic response of homeotherms exposed to cold. In intact white rats, the oxygen consumption can at least triple within minutes after exposure to very cold environments (6). In the warm-acclimated rat exposed to cold the increase in metabolism is dependent almost entirely on muscular activity

such as shivering (9). However, in the functional absence of abdominal viscera the immediate metabolic response to cold exposure is much smaller than that seen in the non-eviscerated warm-acclimated rat (4).

Cold-acclimated rats can raise their oxygen consumption in the cold with little (9) or no (3) shivering and this chemical regulation of heat production is not entirely dependent on the presence of the abdominal viscera (4), at least for the early response. From experiments on cold-acclimated rats, Hsieh and Carlson (10) have suggested *l*-noradrenaline rather than adrenaline as the mediator in chemical regulation of heat production on the basis of the close similarity between the metabolic effects of *l*-noradrenaline and cold exposure. *l*-Noradrenaline had little effect on the oxygen consumption of the warm-acclimated rat while it could more than double that of the cold-acclimated rat without any increase in plasma glucose concentration. In the process of acclimation to cold the rat gradually becomes sensitive to noradrenaline (unpublished observations).

The intimate mechanism of the readjustment of cellular metabolism to the higher levels observed during cold exposure or *l*-noradrenaline treatment is not known. It is not directly known whether specific pathways of metabolism are favored, or whether the normal metabolism of the tissue involved is proportionately increased to the new level of oxygen consumption. The work of Kayser (12) on the respiratory quotient of pigeons, guinea pigs, and rats exposed to moderate cold environments has been taken to indicate a strict increase in fatty acid oxidation in these conditions, but confirmation of the interpretation awaits demonstration of chemical regulation in the pigeon and guinea pig and more direct observations of fatty acid utilization in animals exposed to cold.

The present results on cold-exposed anesthetized rats confirm our previous observation of maintenance of plasma glucose concentration in normal warm- and cold-acclimated rats exposed to $-5°$ C for intervals up to 2.5 hours (7) where essentially normal values for whole blood glucose were obtained throughout the exposure. That this result is due to the inability of the barbital-anesthetized rat to react to normally hyperglycemic agents, as has been suggested by Peterson (14), is negated by the sharp increase in concentration of plasma glucose observed during asphyxia in an anesthetized rat (Fig. 1), and after adrenaline infusion (unpublished observations).

The greater rate of fall of specific activity of plasma glucose in the warm-acclimated rats exposed to cold, which was not apparent in the similarly treated cold-acclimated rats is interpreted as the result of an increase in turnover of plasma glucose associated with the cold exposure. At constant concentration of glucose this is directly related to an increased flow of glucose into the plasma (from the liver or other sources) balanced by an increased uptake of glucose from the plasma by the peripheral tissues. Explanation of this difference in behavior of the plasma glucose pool in warm- and cold-acclimated rats exposed to cold is not immediately apparent. The eventual metabolic fate of the extra glucose going through the plasma pool in shivering warm-acclimated rats is also undetermined.

Carbohydrate metabolism has classically been emphasized in relation to muscle metabolism. Thus, an increased turnover of glucose would be expected in the warm-acclimated rats which shiver on exposure to cold, but not necessarily in the cold-acclimated rats which produce heat by other means than muscular activity. However, recent evidence points to the major role of lipids in the oxidative metabolism of resting muscle in man (1). Also, forced work in anesthetized rats is associated not only with an increased rate of oxidation of glucose but with a proportionately larger increase in oxidation rate of other substrates (11), thus indicating that glucose is not a preferred substrate for muscular activity. This evidence shows that the difference in increase of turnover of plasma glucose in warm- and cold-acclimated rats exposed to cold are not presently explainable on the basis of the different physiological mechanisms of heat production already demonstrated in the two groups of rats. However, since this increased turnover could presumably not take place in the functionally eviscerated warm-acclimated rat, its absence could explain the inadequate, immediate metabolic response of the warm-acclimated rats on exposure to cold (4).

Acknowledgment

The author is indebted to Dr. J. W. Hopkins for statistical appraisal of the data and to Mr. V. Secours for expert technical assistance.

References

1. ANDRES, R., CADER, G., and ZIERLER, K. L. J. Clin. Invest. **35**, 671 (1956).
2. BAKER, N., SHIPLEY, R. A., INCEFY, G., CLARK, R., and SKINNER, S. M. Federation Proc. **14**, 7 (1955).
3. COTTLE, W. H. and CARLSON, L. D. Proc. Soc. Exptl. Biol. Med. **92**, 845 (1956).
4. DEPOCAS, F. Can. J. Biochem. and Physiol. **36**, 691 (1958).
5. DEPOCAS, F. and HART, J. S. J. Appl. Physiol. **10**, 388 (1957).
6. DEPOCAS, F., HART, J. S., and HÉROUX, O. J. Appl. Physiol. **10**, 393 (1957).
7. DEPOCAS, F., MACLEOD, G. K., and HART, J. S. Rev. can. biol. **16**, 83 (1957).
8. GEY, K. F. Biochem. J. **64**, 145 (1956).
9. HÉROUX, O., HART, J. S., and DEPOCAS, F. J. Appl. Physiol. **9**, 399 (1956).
10. HSIEH, A. C. L. and CARLSON, L. D. Am. J. Physiol. **190**, 243 (1957).
11. INGLE, D. J., MORLEY, E. H., and STETTEN, D., JR. Am. J. Physiol. **182**, 263 (1955).
12. KAYSER, C. Compt. rend. soc. biol. **126**, 1219 (1937).
13. NELSON, N. J. Biol. Chem. **153**, 375 (1944).
14. PETERSON, J. McI. Physiol. Revs. **14**, 586 (1934).
15. WALLENFELS, K., BERNT, E., and LIMBERG, G. Angew. Chem. **65**, 581 (1953).

SILICA METABOLISM IN GUINEA PIGS[1]

F. Sauer, D. H. Laughland, and W. M. Davidson

Abstract

The excretion of silica $(SiO_2)_n$ in urine and feces was determined following the oral and intraperitoneal administration of silica to guinea pigs. The urinary excretion of silica after oral administration generally reached a maximum value that was not exceeded by increasing the dose. The urinary excretion after intraperitoneal administration was considerably greater than after oral administration. The results of these experiments suggested that orally and intraperitoneally administered silica is depolymerized to the soluble form prior to excretion in the urine.

Introduction

The economic losses resulting from silica urolithiasis in Western range steers have been recognized for some time (1). A recent report by Connell *et al.* (2) described the incidence and geographical distribution of this condition in Canada. X-ray diffraction patterns and chemical analyses carried out in this division (3) and elsewhere (1) have shown that the calculi obtained from these animals were composed primarily of amorphous silica and organic matter.

The effects of various diets on silica excretion in urine have been reported by King *et al.* (4, 5), who noted that urinary silica excretion was much greater in herbivore than in carnivore. An increased urinary silica excretion was observed following the oral administration of powdered quartz or freshly precipitated silicic acid to dogs (6). Although the amount of silica excreted in the urine was readily influenced by diet these changes were never reflected in the blood levels and it was suggested that animals possess a very low renal threshold for silica.

King (7) studied the urinary excretion of silica following the intraperitoneal injection of either quartz dust or amorphous silica to cats and noted that the excretion of extra silica was transitory and in no instance lasted more than 3 or 4 days. Only a small portion of the administered silica was recovered. Holt (8), using mice, studied the urinary excretion of silica following the intraperitoneal injection of cement, quartz, carborundum, and molding sand. The administration of cement resulted in a definite increase in silica excretion that lasted for 3 or 4 days. Holt (9, 10) studied the distribution of labelled silicon in rat tissues following the intraperitoneal injection of radioactive silica sols. It was noted that alkaline sols were rapidly absorbed from the peritoneal cavity and deposited in the liver and kidneys. Acid sols, however, were poorly absorbed, presumably because of rapid polymerization of silica in the pH range of 5.5 to 6.0.

[1]Manuscript received August 11, 1958.
Contribution No. 409, Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa. A preliminary report of this work was given at the annual meeting of the Chemical Institute of Canada at Toronto in May, 1958.

Scheel *et al.* (11) studied the urinary silica excretion following the intraperitoneal injection of silica gel and sodium silicate into rats. Following the intraperitoneal injection of 100 mg of silica gel, the urine silica excretion increased from 0.2 mg per day to 0.9 mg per day and was still elevated 30 days later. It was noted that the silica content of urine could exceed 0.016% without forming a colloidal suspension.

The determination of silica levels in biological material has been greatly facilitated through the use of an improved silicomolybdate colorimetric technique (12). The interference of phosphorus is minimized in this method by reducing the silicomolybdate complex in 2 *N* sulphuric acid. A useful aspect of the silicomolybdate reaction is that it permits differentiation between 'soluble' silica and 'insoluble' or colloidal silica (13). This was also reported by Weitz *et al.* (14), who noted that molybdate reacted with monosilicic acid at 20° C. in 75 seconds, disilicic acid in 10 minutes, higher oligo acids in 1 hour, and the remainder not at all. Alexander (15) extended this study to include reaction rates.

In the present work a study of silica metabolism in guinea pigs has been undertaken as part of a general project related to silica urolithiasis in steers.

Materials and Methods

The following silicates were used:

1. Sodium metasilicate ($Na_2SiO_3.5H_2O$) obtained from Fisher Scientific Company Limited, Montreal.

2. Precipitated silicic acid ($H_2SiO_3.nH_2O$) obtained from Fisher Scientific Company Limited, Montreal.

3. A 30% silica sol with average particle size of 250 Å supplied by Monsanto (Canada) Limited, Montreal.

4. Tetraethyl orthosilicate obtained from Matheson, Coleman & Bell, Division of The Matheson Company Inc., East Rutherford, New Jersey.

The experiments were carried out with groups of guinea pigs raised from eighth generation inbred stock. The animals were fed a diet of soybean oil meal supplemented with vitamins and minerals (16). The silica content of the diet was 0.75 ± 0.023* mg SiO_2 per gram of feed.

Five guinea pigs were used for each experiment. The animals were kept in plastic metabolism cages and excreta collected in polyethylene containers. Forty-eight-hour urine and fecal collections were analyzed for silica.

The colorimetric technique of King *et al.* (12) was used for silica analysis in these experiments. There was no phosphate interference when phosphorus concentrations remained below 4.8 mg per 100 ml in the final dilution. The mean of eight recoveries obtained when silicates were added to urine or fecal samples was $101 \pm 1.16\%$.* Sodium fluosilicate standards were used in this work because results were reproducible and followed Beer's law with silica

*Standard error of the mean.

concentrations of 0.04 to 0.24 mg per 100 ml. Absorbancy values were determined in a Beckman Model B spectrophotometer at a wavelength of 812 mμ.

Silica analyses on urine samples were generally done in duplicate. An aliquot was analyzed for soluble silica and hydrochloric acid (6 *N*) was added to the remainder in order to dissolve the carbonate precipitates. This acid urine was then adjusted to a known volume and an aliquot slowly dried in a platinum crucible. The residue was ashed, fused with sodium carbonate, and dissolved in distilled water. The dissolved melt was acidified to Congo red with 6 *N* HCl, diluted as required, treated with ammonium molybdate, and reduced in 2 *N* H_2SO_4 with 1,2,4-amino naphthol sulphonic acid reducing agent.

More than 100 urine samples were analyzed in duplicate using both methods and it was found that with concentrations of silica up to 0.0184% there was no significant difference between total and 'soluble' silica ($P>0.05$). At concentrations above 0.0184% the difference between total and 'soluble' silica was significant ($P<0.01$). These data indicate that although the solubility of silica in urine is greater than the reported solubility of silica in water (11, 17, 18), polymerized silica may be present when the concentration of silica in urine exceeds the solubility of silica in water.

The 48-hour fecal collections were ashed, weighed, thoroughly mixed, and a 50-mg sample weighed into a platinum crucible and fused with 3 g sodium carbonate. The silica analyses were carried out as described above.

Results

The urinary excretion of silica in five guinea pigs on the soybean oil meal diet was 2.1 ± 0.28, 2.0 ± 0.21, and 1.5 ± 0.28* mg SiO_2 respectively for three consecutive 48-hour periods.

TABLE I

Urinary SiO_2 excretion following oral administration of (A) single dose sodium silicate (80.0 mg SiO_2); (B) four doses of sodium silicate (80.0 mg SiO_2) at 48-hour intervals

Period	Total SiO_2 excretion (mg)	
	A	B
1	2.7 ± 0.11* (5)†	1.5 ± 0.16* (5)†
2	9.7 ± 0.86 (5)	10.2 ± 0.43 (5)
3	5.7 ± 0.75 (5)	10.3 ± 0.64 (5)
4	3.9 ± 0.65 (5)	10.3 ± 0.82 (5)
5	3.1 ± 0.44 (5)	10.3 ± 0.49 (5)
6		5.9 ± 0.87 (4)
7		5.0 ± 0.60 (4)
8		4.0 ± 0.60 (4)

*Standard error of the mean.
†Number of animals.

TABLE II

Urinary silica excretion following the intraperitoneal injection of a 2% silica sol* (A) 100 mg SiO_2; (B) 60 mg SiO_2

Period	Total SiO_2 excretion (mg)	
	A	B
1	2.2 (3)†	1.9* (2)†
2	28.0 (3)	37.1 (2)
3	17.3 (1)	3.5 (2)
4	11.0 (1)	2.5 (2)

*Prepared from a solution of sodium silicate and dilute HCl.
†Number of animals.

TABLE III

Urinary silica excretion following oral administration of (A) 1% silica sol* (93.1 mg SiO_2); (B) 5% silica sol* (567.0 mg SiO_2)

Period	Total SiO_2 excretion (mg)	
	A	B
1	2.9 ± 0.30† (4)‡	2.0 ± 0.14† (5)‡
2	3.9 ± 0.15 (5)	5.5 ± 0.67 (5)
3	3.6 ± 0.32 (5)	4.0 ± 0.32 (5)
4	3.0 ± 0.31 (5)	3.5 ± 0.31 (5)
5		3.1 ± 0.22 (5)
6		2.9 ± 0.13 (5)
7		3.3 ± 0.22 (5)

*Prepared by diluting a commercial silica sol.
†Standard error of the mean.
‡Number of animals.

TABLE IV

Urinary silica excretion following the intraperitoneal injection of 10 ml of 4% silica sol* (438.8 mg SiO_2)

Period	Total SiO_2 excretion (mg)
1	3.6 ± 0.58† (5)‡
2	50.8 ± 4.21 (5)
3	46.6 ± 1.41 (4)
4	40.1 ± 4.41 (4)
5	33.1 ± 0.25 (3)
6	25.0 ± 3.69 (3)
7	20.3 ± 2.49 (3)
8	13.5 ± 1.96 (3)
9	9.1 ± 1.28 (3)
10	6.3 ± 0.13 (3)

*Prepared by diluting a commercial silica sol.
†Standard error of the mean.
‡Number of animals.

TABLE V

Urinary silica excretion following (A) oral and (B) intraperitoneal administration of 10 ml of a precipitated silicic acid suspension (390 mg SiO_2)

Period	Total SiO_2 excretion (mg)	
	A	B
1	2.3 ± 0.26* (4)†	1.6 ± 0.20* (3)†
2	5.5 ± 0.50 (5)	36.2 ± 3.49 (3)
3	7.1 ± 0.69 (5)	26.6 ± 0.78 (3)
4	5.7 ± 0.43 (5)	24.2 ± 1.06 (3)
5	4.1 ± 0.19 (5)	15.4 ± 0.79 (3)
6	3.3 ± 0.36 (5)	17.0 ± 1.87 (3)
7	2.7 ± 0.27 (5)	13.4 ± 1.11 (3)
8		11.8 ± 1.85 (3)
9		9.2 ± 1.02 (3)
10		8.2 ± 1.23 (3)
11		5.1 ± 0.89 (3)
12		5.1 ± 0.89 (3)
13		4.9 ± 0.75 (3)
14		3.8 ± 0.61 (3)
15		3.3 ± 0.60 (3)

*Standard error of the mean.
†Number of animals.

TABLE VI

Urinary silica excretion following the feeding of (A) pelleted Western hay diet containing 4.65% SiO_2; (B) pelleted soybean oil meal diet containing 4.68% SiO_2

Period	Total SiO_2 excretion (mg)	
	A	B
1	13.4 ± 1.17* (5)†	15.8 ± 1.39* (5)†
2	15.0 ± 1.28 (5)	15.4 ± 1.19 (5)
3	14.9 ± 0.82 (5)	17.8 ± 1.91 (5)
4	13.5 ± 0.87 (5)	15.7 ± 1.89 (5)
5	14.2 ± 0.93 (5)	18.8 ± 2.79 (5)
6		14.6 ± 1.74 (5)

*Standard error of the mean.
†Number of animals.

The experimental results are recorded in Tables I to VI. The term 'period' is used in this paper to indicate a 48-hour time interval.

The data listed for the first period represent the normal silica excretion of the particular groups of animals on test. All silica doses were administered at the end of the first period unless stated otherwise.

Table I, column A, shows the silica excretion following the oral administration of a dose of sodium silicate containing 80.0 mg SiO_2. The urinary silica excretion rose to a maximum of 9.7 ± 0.86* mg 48 hours after dosing

*Standard error of the mean.

and returned to the normal level after 8 days. The term 'net silica excretion' is used in this paper to indicate the difference between the silica ingested and the silica excreted in the urine and feces. This amounted to 48.3 ± 3.58* mg SiO_2 in this experiment and represented approximately 60% of the administered dose.

Table I, column B, shows the results of administering four successive doses of sodium silicate at 48-hour intervals, each containing 80.0 mg SiO_2. The urinary silica excretion reached a maximum of 10.3 mg per 48-hour period and did not increase with subsequent doses. The experiment was terminated at period 8 when the urinary silica excretion approached the normal value. The net silica excretion was 307.0 ± 7.85* mg, or approximately 96% of the administered dose.

These experiments suggested that there might be a well-defined limit to silica absorption from the intestinal tract. Therefore, in the next experiment five guinea pigs were injected intraperitoneally with a 2% silica sol of pH 5.0 prepared from sodium silicate. The animals were injected with 3- and 5-ml doses containing 60 and 100 mg of SiO_2 respectively. The results of this experiment (Table II) indicated that the 48-hour urinary silica excretion was approximately three times as great as that obtained in the previous two experiments.

Table III shows the urinary silica excretion following the feeding of a silica sol with an average particle size of 250 Å. The undiluted sol gelled rapidly at a pH below 9, but gelling did not occur at any pH if the sol were first diluted to a concentration of 5% or less.

The urinary silica excretion did not increase appreciably after the oral administration of this sol in doses equivalent to 93.1 and 567.0 mg SiO_2 respectively. The urinary silica was present entirely in the 'soluble' or 'molybdate reactive' state, indicating that silica particles of the size present in the sol were not excreted without a prior depolymerization to 'soluble' silica. With the group receiving the 5% silica sol containing 567.0 mg SiO_2, the net silica excretion was 487.2 ± 21.57* mg SiO_2, or approximately 86% of the administered dose.

A large increase in urinary silica excretion was obtained after an intraperitoneal injection of a silica sol containing 438.8 mg SiO_2. The results recorded in Table IV show that the 48-hour urinary silica excretion at period 2 reached a maximum of 50.8 ± 4.21* mg and returned approximately to the normal level at the end of period 10. The net silica excretion in this experiment was 277.5 ± 20.99* mg SiO_2. This represented 63% of the injected dose.

The results recorded in Table V were similar to those described above except that precipitated silicic acid of undetermined particle size was used instead of the silica sol. The urinary silica levels obtained following the oral administration of this precipitated silicic acid (Table V) were similar to those recorded with the silica sol (Table III). The net silica excretion in

*Standard error of the mean.

this experiment was 251.2 ± 25.80* mg at the end of period 7. This represented approximately 64% of the administered dose of 390 mg SiO_2. This relatively low recovery may have been due to silica retention in the intestinal tract.

The urinary silica excretion following the intraperitoneal injection of a 5% precipitated silicic acid suspension containing 390 mg SiO_2 is recorded in Table V. The urinary silica excretion as measured at 48-hour intervals reached a maximum of 36.2 ± 3.49* mg and subsequently dropped to normal values after 28 days. The results from two of the animals were not included in the table. Their urinary silica excretion rose to a maximum of 13.4 mg and remained high for 48 days. The net silica excretion of the entire group was 187.0 ± 15.16* mg. This accounted for approximately 48% of the injected dose. It is probable that the rest of the injected silica was retained by the tissues.

There was no well-defined increase in silica content of the fecal ash following the intraperitoneal injection of silica. The question of silica excretion into the intestinal tract following intraperitoneal injection requires further study.

Table VI shows the urinary silica levels obtained after feeding a pelleted diet of Western hay to guinea pigs. This diet, supplemented with vitamins, minerals, and casein, contained 4.65 g SiO_2 per 100 g air-dried material. Over a 10-day period silica excretions during successive 48-hour intervals ranged from 13.4 ± 1.17* mg to 15.0 ± 1.28* mg SiO_2. During the same 48-hour intervals the feces contained from 1 to 2 g SiO_2. Table VI also shows the urinary silica excretion following the feeding of a pelleted soybean oil meal diet containing silica gel. This diet contained 4.68 g SiO_2 per 100 g air-dried material. The silica excretion during 48-hour intervals over a 10-day period ranged between 14.6 ± 1.74* mg and 18.8 ± 2.79* mg.

Discussion

In this study urine samples were analyzed for total and soluble silica. The results showed that silica in guinea pig urine does not polymerize at concentrations less than 0.018%. The method for "soluble" silica, however, could not be applied when the concentration of silica exceeded 0.018% because polymerization occurred in some samples. Although these results indicated that the solubility of silica in urine approximates the reported solubility of silica in water (11, 17, 18), some of the urine samples that were analyzed contained quantities of "soluble" silica far in excess of those present in aqueous systems.

In the experiments listed in Tables I and II a solution of sodium silicate was administered orally. Although a gel was probably formed on contact with the hydrochloric acid of the stomach, higher urinary silica levels resulted from the feeding of this material than were obtained with comparable doses of other forms of inorganic silica.

*Standard error of the mean.

The results showed that silica absorption and excretion following a single oral dose of sodium silicate reached a maximum within 48 hours and gradually returned to normal values after 8 days. The experiment recorded in Table I, column B, was carried out in order to determine if the silica excretion could be increased by administering four doses of sodium silicate at 48-hour intervals. The results showed that the increase did not occur and that the excretion over a 48-hour period did not exceed 10.3 mg. The urinary excretion of silica was apparently limited by restricted absorption from the gastrointestinal tract and not by the blood transport mechanism or kidneys since in the succeeding experiment (Table II) a threefold increase in silica excretion followed the intraperitoneal injection of a comparable silica dose. Apparently the kidneys are capable of excreting much larger amounts of silica than are normally absorbed from the gastrointestinal tract.

The results in Tables III and V showed that only small increases in silica excretion followed the oral administration of the silica sol and the precipitated silicic acid. Since in these experiments all of the silica in the urine was in the 'soluble' or 'molybdate reactive' form it appears that the silica particles underwent depolymerization prior to excretion.

From these results the possibility may be considered that the absorption and urinary excretion of the ingested silica is limited by its solubility in the contents of the gastrointestinal tract. This was supported by later work which showed that when guinea pigs were dosed with 0.96 ml of tetraethyl orthosilicate in 2.0 ml of ethanol, urinary excretion values in excess of 40 mg per 48 hours were obtained.

Comparative data are given in Tables IV and V for the urinary excretion of silica after the intraperitoneal injection of a silica sol or a precipitated silicic acid. A marked increase in the excretion of urinary silica followed the use of the intraperitoneal instead of the oral route of administration. The urinary silica excretion after the intraperitoneal injection of the silica sol was increased above normal for 18 days and at that time 63% of the administered silica was accounted for. The urinary silica excretion after the intraperitoneal injection of precipitated silicic acid was increased above normal for 28 days and at that time 48% of the administered silica was recovered. Although some of the silica in the urine polymerized at the higher concentrations, the greater part was present in the soluble form indicating that the injected silica particles were depolymerized prior to excretion.

The reason for the greater silica excretion following intraperitoneal injection is not definitely known. These results indicate that conditions in the peritoneal cavity favor the depolymerization and subsequent excretion of silica.

The results in Table VI indicate the urinary silica excretion obtained when animals were fed pelleted diets of either Western hay (4.65% SiO_2) or silica-supplemented soybean oil meal (4.68% SiO_2). These animals ingested approximately 2.0 g silica during each 48-hour period. The results show that when large quantities of either silica gel or plant silica were ingested,

the amount of urinary silica excreted was comparable with that obtained with much smaller doses of sodium silicate. The results indicate that when inorganic silica is fed to guinea pigs, the silica excretion in urine, during a 48-hour period, approaches a maximum. The amount of silica required to produce this maximum depends primarily on the solubility of the administered silicate.

Acknowledgments

The authors wish to extend thanks to Mr. P. M. Richard for his assistance with the laboratory animals and to Monsanto (Canada) Ltd. for their generous supply of the silica sol used in these experiments.

References

1. SWINGLE, K. F. Am. J. Vet. Research, **14**, 493 (1953).
2. CONNELL, R., WHITING, F., and FORMAN, S. A. Can. J. Comp. Med. Vet. Sci. In press. 1958.
3. FORMAN, S. A., WHITING, F., and CONNELL, R. Can. J. Comp. Med. Vet. Sci. In press. 1958.
4. KING, E. J., STANTIAL, H., and DOLAN, M. Biochem. J. **27**, 1002 (1933).
5. KING, E. J. and BELT, T. H. Physiol. Revs. **18**, 329 (1938).
6. KING, E. J., STANTIAL, H., and DOLAN, M. Biochem. J. **27**, 1007 (1933).
7. KING, E. J. Biochem. J. **32**, 426 (1938).
8. HOLT, P. F. Brit. J. Ind. Med. **7**, 12 (1950).
9. HOLT, P. F. Brit. J. Exptl. Pathol. **35**, 52 (1954).
10. HOLT, P. F. and YATES, D. M. Biochem. J. **54**, 300 (1953).
11. SCHEEL, L. D., FLEISCHER, E., and KLEMPERER, F. W. A. M. A. Arch. Ind. Hyg. and Occupational Med. **8**, 564 (1953).
12. KING, E. J., STACY, B. D., HOLT, P. F., YATES, D. M., and PICKLES, D. Analyst, **80**, 441 (1955).
13. DIENERT, F. and WANDENBULCKE, F. Compt. rend. **176**, 1478 (1923).
14. WEITZ, E., FRANCK, H., and SCHUCHARD, M. Chemiker-Ztg. **74**, 255 (1950).
15. ALEXANDER, G. B. J. Am. Chem. Soc. **75**, 5655 (1953).
16. WESSON, L. G. Science, **75**, 339 (1932).
17. ALEXANDER, G. B., HESTON, W. M., and ILER, R. K. J. Phys. Chem. **58**, 453 (1954).
18. GOTO, K. and OKURA, T. Kagaku (Tokyo), **23**, 426 (1953).

THE PREPARATION OF SUCROSE-C^{14} WITH HIGH SPECIFIC ACTIVITY AND HIGH TRACER YIELD USING DETACHED SUGAR BEET LEAVES[1]

C. D. Nelson and D. C. Mortimer

Abstract

Radioactive sucrose, in millicurie amounts, was prepared by feeding a detached sugar beet leaf $C^{14}O_2$ in air for 15 to 20 minutes followed by 5 to 20 minutes additional photosynthesis in air. The low endogenous sucrose in the leaf coupled with the improved tracer yield effected by the displacement technique gave sucrose with high specific activity. This sucrose-C^{14}, hydrolyzed by 1% invertase, was a good source for radioactive glucose and fructose. Paper chromatographic procedures were used for the isolation and purification of the sugars.

Introduction

Commercially available C^{14}-labelled sucrose is prepared biologically by some variation of the method (9) of feeding a plant $C^{14}O_2$ in the light. The radioactive sucrose produced is isolated as a mixture with the endogenous non-radioactive sucrose. If the plant is a sucrose accumulator, the radioactive product will have a relatively low specific activity. On the other hand a plant which has a low endogenous sucrose content may convert a small proportion of the assimilated carbon dioxide to sucrose. In this case the sucrose would have a high specific activity but the tracer yield would be low. For some types of research, such as the study of translocation in plants (8), the sucrose should have a higher specific activity than that commercially available. This need focused attention on the development of a suitable laboratory-scale system for the biological preparation of such radioactive sucrose.

The approach to the problem was suggested by the displacement studies with detached sugar beet leaves (5), which have a small amount of endogenous sucrose. In these studies it had been observed that most of the photosynthetically assimilated $C^{14}O_2$ could be recovered in sucrose if the leaves were allowed to carry out photosynthesis in $C^{12}O_2$ for a few minutes after the completion of the assimilation period in $C^{14}O_2$.[2] After preliminary trials a method was developed for preparing millicurie amounts of sucrose-C^{14}.

Materials and Methods

Sugar beet plants (var. Klein Wanzleben) were grown in the greenhouse in irrigated sand culture as previously described (5).

[1]Manuscript received September 25, 1958.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 4996.

[2]"In $C^{14}O_2$" means in air enriched with $C^{14}O_2$; "in $C^{12}O_2$" means in air with its normal $C^{12}O_2$ content.

A sugar beet leaf (about 2 g fresh weight) was excised from a plant and put in a transparent plastic chamber (10). This chamber was incorporated into a closed circuit of rubber tubing containing the following additional components: (1) a Sigmamotor pump, (2) a housing containing a thin mica window (1.2 mg per cm^2) Geiger–Müeller tube attached to a laboratory rate meter, (3) a generator for releasing $C^{14}O_2$ from a solution of $Na_2C^{14}O_3$, and (4) a reservoir for thorough mixing of the gas. The total volume of the closed system could be varied between 0.75 and 1.5 liters. Light from incandescent lamps was passed through a water filter to give an illumination of 2000 ft-c at the surface of the leaf. At zero time $C^{14}O_2$ (0.1 to 1.0 mc, specific activity 0.74 mc per mg C) was introduced into the system by acidifying the carbonate in the generator. When the rate meter reading indicated that at least 95% of the tracer carbon had been incorporated into the leaf, the chamber was opened and the leaf allowed to carry on photosynthesis in air for 4 to 20 minutes.

The leaf was killed by maceration in 150 ml of hot 80% (w/w) ethanol in a Waring blendor. The macerate was boiled for 30 minutes, filtered, and the residue was washed with hot 80% ethanol. The ethanol-soluble fraction was concentrated *in vacuo* at 40° C to a final volume of about 1 ml. The concentrated extract was used for paper chromatography.

In preliminary experiments using 100 μc of $C^{14}O_2$ the radioactive compounds in the ethanol-soluble extract were separated by two-dimensional ascending paper chromatography and assayed as described by Burma and Mortimer (5). In the preparative experiments using up to 1 mc of $C^{14}O_2$, the sugars were separated from the other compounds of the ethanol-soluble fraction by ascending band chromatography on Whatman No. 3 filter paper using pyridine:ammonium hydroxide:isobutanol (4:2:1) (5). After elution of the sugars with water, sucrose was separated from the hexoses by descending band chromatography on Whatman No. 1 filter paper using double development with *n*-propanol:ethyl acetate:water (7:1:2) (1). Sucrose was eluted from the paper with water and the solution stored at −40° C. Radioactive spots or bands were located on the paper by autoradiography using Kodak No-Screen X-ray film.

Fructose and glucose were obtained by inverting sucrose on Whatman No. 1 filter paper with 1% invertase (Wallenstein Laboratories, New York) solution and chromatographing in the propanol solvent.

Sugar concentrations were determined colorimetrically using anthrone (7).

Aliquots of the ethanol-soluble fractions and sucrose solutions were plated in an infinitely thin layer on aluminum planchets for assay of their C^{14} content. Ethanol-insoluble residues were converted to CO_2 by the total combustion method of Baker *et al.* (2) and the C^{14} content was assayed as $BaCO_3$. All determinations were made in triplicate using a methane flow proportional counter. All planchets were counted to a standard error of 3% or less. The counting data obtained from the $BaCO_3$ planchets were corrected for self-absorption (6).

Results and Discussion

Analysis of sugar beet leaves showed free sugars present in the following amounts: sucrose 1.7 mg, glucose 5.3 mg, and fructose 4.7 mg per g fresh weight. This small amount of endogenous sucrose made these leaves ideal for the preparation of sucrose-C^{14} with high specific activity.

Preliminary experiments were carried out in which sugar beet leaves were allowed to carry on photosynthesis for 1 minute in air containing 100 μc of C^{14} followed by photosynthesis in normal air for 5 to 20 minutes. The distribution of radioactivity among the compounds of the ethanol-soluble fraction is shown in Table I. With increasing time of photosynthesis in $C^{12}O_2$, sucrose accumulated up to 90% of the C^{14} in this fraction while the C^{14} content of the phosphate esters and glyceric acid decreased. It was concluded that under these conditions C^{14} was displaced from the photosynthetically labile intermediates and was accumulated in sucrose. It seemed possible, therefore, to use this technique to prepare large amounts of sucrose-C^{14} with a high specific activity.

TABLE I

Distribution of C^{14} among the compounds of the ethanol-soluble fraction of sugar beet leaves after photosynthesis for 1 minute in 100 μc of $C^{14}O_2$ followed by photosynthesis in $C^{12}O_2$

Compounds	Conditions for photosynthesis		
	$C^{14}O_2$, 1 min (%)	$C^{14}O_2$, 1 min $C^{12}O_2$, 5 min (%)	$C^{14}O_2$, 1 min $C^{12}O_2$, 20 min (%)
Diphosphates	13.0	2.4	0.6
G-1,2-P	4.2	1.3	
Hexose monophosphates	0.8		
Phosphoglyceric acid	1.6	1.0	
Phosphoenolpyruvic acid	0.5		
Malic acid	3.5	5.0	6.0
Aspartic, glutamic acids	1.9	1.5	2.0
Glycine, serine	36.0	9.3	1.0
Alanine, threonine	8.0	0.4	
Glyceric acid	7.9	1.2	
Glycolic acid	1.3		
Sucrose	21.3	77.9	90.4

Tables II and III show the results of two experiments where larger amounts of $C^{14}O_2$ (782 and 873 μc) were administered. About 2 g of leaf tissue was used in these experiments. Since the rate at which leaves fix carbon dioxide during photosynthesis varies from leaf to leaf and also with the concentration of carbon dioxide offered, the uptake was followed on the rate meter until at least 95% of the $C^{14}O_2$ had been fixed. Table III shows that the percentage of C^{14} incorporated into the 80% ethanol-soluble fraction was about the same in the two experiments. However, 1.5 times more C^{14} was incorporated into sucrose when photosynthesis in air was continued for a longer period of time.

TABLE II

Uptake of $C^{14}O_2$ by sugar beet leaves in photosynthesis and specific activities of the sugars isolated

	Fresh weight of leaf, g	Total C^{14} offered, μc	Total C^{14} taken up:		Specific activity (μc per mg C)		
			μc	%	Sucrose	Glucose from sucrose	Fructose from sucrose
Experiment 1 (17 min in $C^{14}O_2$, 0.3%; 4 min in $C^{12}O_2$)	1.7	782	750	96	54.7	59.0	53.7
Experiment 2 (24 min in $C^{14}O_2$, 0.2%; 7 min in $C^{12}O_2$)	2.0	873	861	99	49.2	52.4	46.8

TABLE III

Distribution of C^{14} in various fractions of the sugar beet leaves described in Table II

Fraction	Experiment 1, total activity:		Experiment 2, total activity:	
	μc	%	μc	%
Ethanol soluble	447	60	553	64
(Sucrose)	(255)	(34)	(450)	(52)
(Remainder)*	(192)	(26)	(100)	(12)
Ethanol insoluble	303	40	308	36
Totals	750	100	861	100

*Obtained by difference, checked by direct assay.

The specific activity data (Table II) show that the fructose moiety of sucrose contained less C^{14} than the glucose moiety when the leaves were killed following photosynthesis in $C^{12}O_2$. Benson (3) has reported a similar result in short-term photosynthesis with soybean; the fructose moiety of sucrose was more radioactive than the glucose moiety when the leaves were killed following photosynthesis for 15 or 60 seconds in $C^{14}O_2$. Burma and Mortimer (4) have found that sucrose is formed from uridine diphosphate glucose (UDPG) and fructose-6-phosphate (F-6-P) in sugar beet leaves. If the pool of F-6-P was smaller than the pool of UDPG the unequal labelling in the two moieties of sucrose can be explained.

This method for preparing sucrose-C^{14} has become standard practice in our laboratory. As well as giving a product of high specific activity the whole preparation takes very little time. By starting with $C^{14}O_2$ of higher specific activity the specific activity of sucrose isolated could be increased further. Moreover, using leaves of other species it may be possible to increase the yield of tracer in compounds other than sucrose by the technique of displacement of C^{14} from photosynthetically labile compounds.

References

1. ALBON, N. and GROSS, D. Analyst, **77**, 410 (1952).
2. BAKER, N., FEINBERG, H., and HILL, R. Anal. Chem. **26**, 1504 (1954).
3. BENSON, A. A. Arch. Biochem. Biophys. **32**, 223 (1951).
4. BURMA, D. P. and Mortimer, D. C. Arch. Biochem. Biophys. **62**, 16 (1956).
5. BURMA, D. P. and MORTIMER, D. C. Can. J. Biochem. and Physiol. **35**, 835 (1957).
6. KARNOVSKY, M. L., FOSTER, I. M., GIDEZ, L. I., HAGERMAN, D. D., ROBINSON, C. V., SOLOMON, A. K., and VILLEE, C. A. Anal. Chem. **27**, 852 (1955).
7. MORRIS, D. L. Science, **107**, 254 (1948).
8. NELSON, C. D. and Gorham, P. R. Can. J. Botany, **35**, 703 (1957).
9. PUTMAN, E. W., HASSID, W. Z., KROTKOV, G., and BARKER, H. A. J. Biol. Chem. **173**, 785 (1948).
10. TOWERS, G. H. N. and MORTIMER, D. C. Can. J. Biochem. and Physiol. **34**, 511 (1956).

PREPARATION AND ASSAY OF D-ERYTHROSE 4-PHOSPHATE[1]

J. N. BAXTER,[2] A. S. PERLIN, AND F. J. SIMPSON

Abstract

The oxidation of D-glucose 6-phosphate by lead tetraacetate in cold acetic–propionic acid produces D-erythrose 4-phosphate contaminated with a small proportion of D-glyceraldehyde 3-phosphate. The product is a suitable substrate for certain enzymic studies, as illustrated by its use in the assay of transaldolase and transketolase. D-Erythritol 4-phosphate may readily be obtained in high yield by reducing the crude oxidation mixture with sodium borohydride. Oxidation of D-fructose 6-phosphate with 1 mole of lead tetraacetate gives chiefly D-erythrose 4-phosphate, whereas oxidation with 2 moles produces mainly D-glyceraldehyde 3-phosphate. The stability of D-erythrose 4-phosphate under a variety of conditions was also examined.

Introduction

D-Erythrose 4-phosphate (E-4-P),* an important intermediary metabolite (1, 2), has been synthesized by Ballou, Fischer, and MacDonald (3). In this elegant procedure D-erythrose is converted in several stages into the crystalline cyclohexylammonium salt of the dimethyl acetal of E-4-P, from which solutions of E-4-P may be prepared as required. The present work describes a method by which readily available G-6-P is oxidized with lead tetraacetate to yield E-4-P.

Materials

Lead tetraacetate, obtained commercially from the Matheson Company Inc., was stored moist with acetic acid at 0° C. Sodium glutathione (reduced) and R-5-P were purchased from Schwarz Laboratories Inc., while the Sigma Chemical Company was the source of G-6-P, F-6-P, DPN, DPNH, TPN, and TPNH. Acid phosphatase was purchased from the Worthington Biochemical Corporation.

D-Erythrose was prepared by oxidation of D-glucose with lead tetraacetate (4). S-7-P was prepared enzymatically from R-5-P and obtained as the barium salt after separation on a Dowex-1 formate column (5). Xu-5-P was prepared from D-xylulose with a purified xylulokinase from *Aerobacter aerogenes* (6).

[1]Manuscript received September 22, 1958.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. A preliminary account of this work was presented at the annual meeting of the Canadian Society of Microbiologists at Halifax, Nova Scotia, June 18–20, 1958.
Issued as Paper No. 257 on the Uses of Plant Products and as N.R.C. No. 5004.
[2]National Research Council of Canada Postdoctorate Fellow, 1956–1958.
*Abbreviations: DHAP, dihydroxyacetone phosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; E-4-P, D-erythrose 4-phosphate; F-6-P, D-fructose 6-phosphate; α-GP, α-glycerophosphate; G-3-P, D-glyceraldehyde 3-phosphate; G-6-P, D-glucose 6-phosphate; 6-PG, 6-phospho D-gluconic acid; PGA, 3-phospho D-glyceric acid; R-5-P, D-ribose 5-phosphate; S-7-P, D-sedoheptulose 7-phosphate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; Xu-5-P, D-xylulose 5-phosphate.

Crystalline aldolase and G-3-P dehydrogenase were prepared from rabbit muscle by the method of Cori *et al.* (7). The ammonium sulphate fraction collected between 20 and 50% saturation was used as the source of hexosephosphate isomerase, and the fraction between 52 and 70% saturation, after recutting between 55 and 65% saturation, as the source of triosephosphate isomerase and α-GP dehydrogenase (8). G-6-P dehydrogenase was purified by Dr. B. K. Bhuyan of this laboratory by the method described by Kornberg and Horecker (9). Transketolase, which did not contain D-ribulose 5-phosphate 3-epimerase, was purified 20 fold from an acetone powder of pork liver (6).

Transaldolase was prepared from a local brewer's yeast by the method of Horecker and Smyrniotis (10) with some minor modifications. With this local yeast, the maximum amount of transaldolase was extracted with 0.4 *M* sodium bicarbonate rather than with 0.1 *M* as recommended. The extract contained an active oxidase that attacked both DPNH and TPNH in a reaction mixture containing 20 micromoles (μM) of triethanolamine buffer and 0.06 μM of reduced pyridine nucleotide per milliliter. This oxidase was inhibited by cyanide (0.01 *M*, 14%; 0.1 *M*, 66% inhibition) and by cysteine (0.01 *M* 35%; 0.1 *M*, 60% inhibition) but not by arsenate, pyrophosphate, glutathione, or acriflavin (11). The reduced pyridine nucleotide oxidase was partially separated, with a considerable loss of transaldolase, during the step with calcium phosphate gel by washing the gel with 0.017 *M* sodium pyrophosphate (adjusted to pH 7.6 with hydrochloric acid) before eluting transaldolase with 0.15 *M* sodium pyrophosphate (adjusted to pH 7.6 with sulphuric acid). Additional oxidase was removed by precipitation with ammonium sulphate (50% saturation) at neutrality, and the last traces at pH 4 (55% saturation). The final dialyzed preparations of transaldolase were free of oxidase and phosphatase. They were stable for months when stored at $-20°$ C, but less stable when stored in the presence of ammonium sulphate. The precipitate obtained at neutrality with ammonium sulphate (50% saturation) was used as a source of reduced pyridine nucleotide oxidase.

Methods

Preliminary studies on the kinetics of oxidations with lead tetraacetate were conducted as follows: An ice-cold solution of the sugar (130 μM) in 60 ml of a 3:1 mixture of acetic and propionic acids containing 1 ml of water and in some runs mineral acid, was mixed at zero time with an ice-cold solution of lead tetraacetate (800 μM) in 15 ml of the acetic–propionic acid mixture. At intervals, 5-ml aliquots were pipetted into a KI reagent and the liberated iodine was determined. The lead tetraacetate consumed was obtained by subtraction of the titer from the titer of a blank.

Analytical Methods

Lead tetraacetate was determined by adding suitable aliquots to 15 ml of KI reagent (10% potassium iodide and 27% anhydrous sodium acetate (12)). The liberated iodine was titrated with 0.1 *N* sodium thiosulphate solution

using a micrometer syringe burette. The amount of lead tetraacetate present in the solution was calculated from the relationship: 1 ml 0.1 N $Na_2S_2O_3$ solution = 50 μM $Pb(OCOCH_3)_4$.

Inorganic phosphate was determined colorimetrically by the method of Fiske and Subbarow (13). Ester phosphate was measured as inorganic phosphate released by hydrolysis at 100° C for 18 hours with 1 N sulphuric acid, except when G-6-P was present, when 10 N sulphuric acid was employed. Erythrose, free or combined, was determined colorimetrically by the sulphuric acid – cysteine method (14).

Enzymatic Methods

Spectrophotometric measurements were made at room temperature with a Beckman model DU spectrophotometer in cuvettes with a light path of 1 cm and a capacity of 1 ml.

G-6-P was determined enzymatically with G-6-P dehydrogenase, and F-6-P likewise but with the addition of hexosephosphate isomerase (15). G-3-P was determined with G-3-P dehydrogenase or with α-GP dehydrogenase (15).

E-4-P was determined by the reaction with F-6-P catalyzed by transaldolase (Fig. 1). The reaction mixture contained, per milliliter, 20 μM of triethanolamine buffer, pH 7.6; 2 μM sodium versenate; 4 μM sodium arsenate; 1 μM F-6-P; 0.25 μM DPN; 5 μM sodium glutathione; 150 μg of thrice-recrystallized G-3-P dehydrogenase; 0.5 units of transaldolase; and from 0.01 to 0.1 μM of E-4-P.

A high concentration of F-6-P was found desirable to drive the reaction to completion. Sodium arsenate could be varied from 2 to 20 μM per milliliter without effect. In addition to triethanolamine, glycylglycine was a satisfactory buffer. Pyrophosphate, ammonium sulphate, and sodium sulphate were inhibitory. At a level of 10 μM per ml ammonium or sodium sulphate caused a 20% decrease and 150 μM per ml of ammonium sulphate inhibited completely. Because of this inhibition, the suspension of crystalline G-3-P dehydrogenase was centrifuged to remove the ammonium sulphate

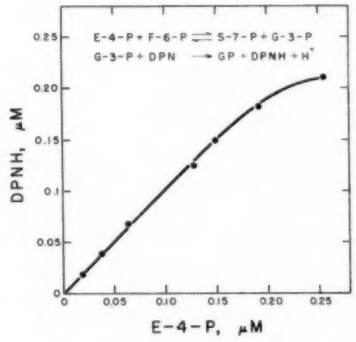


FIG. 1. Standard curve for the determination of E-4-P.

and the crystals were dissolved in 0.05 *M* versene, pH 8.0. Sodium acetate, sodium citrate, sodium phosphate, D-erythrose, and S-7-P were innocuous at levels of 1 μM per ml. Versene was added as a precaution against the accidental carry-over of lead into the E-4-P sample, although repeated tests demonstrated that the omission of versene had no effect on the results and thus indicated the absence of lead.

The reaction was initiated by the addition of E-4-P and the reduction of DPN followed by measuring the change in optical density at 340 mμ. The small increase in the optical density of the reaction mixture caused by the addition of E-4-P was determined in a control from which the enzymes were omitted. The blank without E-4-P had a steady rate of approximately 0.001 units optical density per minute. The reaction with E-4-P proceeded rapidly during the first 3 minutes and then drifted slowly to completion. With 0.02 μM of E-4-P the reaction was complete in 45 minutes whereas with 0.1 μM 120 minutes were required. Changes in optical density were read at intervals until the rate for the reaction mixture equalled that of the control. The slow drift observed was not caused by denaturation of the enzymes, for similar results were obtained when the reaction mixture was allowed to stand for 30 minutes before the addition of E-4-P.

Experimental

Oxidation of G-6-P

Earlier studies (4) demonstrated that D-glucose in acetic acid consumes 2.0 moles of lead tetraacetate per mole, giving D-erythrose diformate, from which D-erythrose may be obtained in high yield by mild hydrolysis. In attempting to extend this oxidation reaction to prepare E-4-P from G-6-P pronounced over-oxidation occurred (Table I) probably because the formate ester groups of the product were unstable. (Over-oxidation has also been observed with uronic acids (16) and D-glucose itself is over-oxidized in the

TABLE I

Lead tetraacetate consumption at O° C (moles/mole)

Carbohydrate	Conditions	Time, minutes					
		10	20	30	60	120	180
Glucose		1.02	1.48	1.79	1.85	2.02	2.05
Glucose	In presence of 1.25 moles sulphuric acid	1.65	1.97	2.02	2.10	2.16	2.24
Glucose	In presence of 1.10 moles phosphoric acid	0.73	1.26	1.52	2.05	2.15	2.21
G-6-P, Ba salt	At 25° C	2.56	2.84	3.20*	—	3.84†	4.46‡
G-6-P, K salt		0.89	1.28	1.46	1.86	2.03†	2.15‡
G-6-P, K salt	In presence of 2.50 moles sulphuric acid	1.56§	—	1.75	1.92	2.00†	2.12‡
F-6-P, K salt		2.06	—	2.16	2.23	2.25	2.32

*After 40 minutes.
†After 90 minutes.
‡After 210 minutes.
§After 15 minutes.

presence of phosphoric acid or sulphuric acid (Table I).) When only 2 moles of lead tetraacetate were added to a solution of G-6-P, much G-6-P was not oxidized and both E-4-P and G-3-P were formed. Klybas *et al.* (17) found that the tetrose- and triose-phosphates were present in the mixture in almost equimolar amounts, and have utilized this fact to synthesize, enzymatically, D-sedoheptulose-1,7-diphosphate.

To increase the relative proportion of E-4-P in the oxidation mixture it was necessary to reduce over-oxidation of G-6-P. Preliminary experiments showed that substantial improvement could be obtained by using the potassium salt of G-6-P at high dilution (1 millimole per liter), in the presence of a low concentration of water (0.2%), and at a temperature of 0° C (Table I).

Preparation of E-4-P

Barium G-6-P heptahydrate (1.04 g) was wetted with acetic acid (1 ml) and dissolved in water (200 ml). Potassium sulphate (348 mg in 10 ml water) was added, the barium sulphate removed by filtration, and the filtrate lyophilized and dissolved in 5 ml of water. The G-6-P content of an aliquot of this solution was determined colorimetrically (13) and checked by enzymic assay with G-6-P dehydrogenase.

An aliquot of the potassium G-6-P solution (0.5 ml containing 200 μM) was added to a mixture of acetic acid (210 ml) and propionic acid (70 ml) which was stirred mechanically and cooled to 0° C. Lead tetraacetate (400 μM) in acetic–propionic acid (10 ml) was added slowly and the opalescent reaction mixture was stirred and maintained at 0° C for 5 hours. The solution was concentrated by freeze-drying, to avoid destruction of products, to about 20 ml (*not to dryness**). More of the solvent was removed by twice adding water (100 ml) and then freeze-drying to 20 ml. After thawing, the solution was passed down a column of Amberlite IR-120 (H form) (3 g, air-dried) which was then washed with 100 ml of water. The eluate and washings were adjusted to pH 3–4 with a solution of 1 *M* ammonium bicarbonate (0.4 ml) and stored for 18 hours at 0° C to complete the hydrolysis of formate esters. The solution was then concentrated by freeze-drying to a volume of about 20 ml, and the product examined as described below.

Examination of the Oxidation Product

An aliquot of the solution was treated with acid phosphatase and the resulting solution examined by chromatography on Whatman No. 1 paper using ethyl acetate – acetic acid – water (9:2:2) as solvent and aniline oxalate spray. Erythrose and a smaller proportion of glyceraldehyde were found, together with a trace of glucose.

Another portion of the product was incubated for 3 hours at 25° C and at pH 7.0 with transaldolase, versene (0.002 *M*), glycylglycine buffer (0.04 *M*), and F-6-P. The solution was adjusted to pH 6 with 0.1 *N* sulphuric acid, heated at 100° C for 5 minutes, and filtered to remove the denatured

*When the solution was concentrated to dryness in the presence of lead acetate the yield of product was lowered and inorganic phosphate produced.

enzyme. The filtrate was incubated for 1 hour at 37° C with acid phosphatase, then treated with Amberlite IR-4B (OH form). Sedoheptulose was detected in the solution by chromatography on Whatman No. 1 paper, using *n*-butanol–ethanol–water (40:11:19) as solvent and orcinol – trichloroacetic acid spray (18); the characteristic blue spot had R_f 0.18, as did an authentic sample of sedoheptulose. Fructose gave a brown spot with R_f 0.20. This experiment indicated that E-4-P was present in the product from oxidation of G-6-P.

Enzymatic assay of a solution prepared from 223 μM G-6-P showed that the product contained 172 μM of E-4-P (77% yield), 37 μM of G-3-P, and 8 μM of G-6-P. Attempts to purify E-4-P by fractional precipitation of the barium salt with methanol failed. Nor could E-4-P and G-3-P be separated satisfactorily on an ion exchange resin column (19). Paper chromatography, with a variety of solvent systems, also proved ineffective. However, E-4-P freed of all but a trace of G-3-P was prepared by a combination of enzymic and chemical methods (see "Assay of Transaldolase").

Preparation of D-*Erythritol 4-Phosphate*

Barium G-6-P heptahydrate (0.98 g) was oxidized with lead tetraacetate, the solution of E-4-P was treated with an excess of sodium borohydride, and the product was isolated according to the procedure of Ballou *et al.* (3) as the cyclohexylammonium salt. The yield of crude crystalline material was 665 mg, and this, after two recrystallizations from hot absolute ethanol, gave 375 mg (50% yield from G-6-P) of the cyclohexylammonium salt of D-erythritol 4-phosphate, m.p. 133–140° C, $[\alpha]_D^{25}$ −2.5° (*c*, 2, water); after acidifying the solution with excess Amberlite IR-120, $[\alpha]_D^{25}$ +2.5° (*c*, 1, water). Literature (20): $[\alpha]_D^{25}$ −2.3° and + 2.6°, respectively. Found: C, 48.2; H, 9.3; N, 6.9. Calc. for $C_{16}H_{37}O_7PN_2$: C, 48.0; H, 9.2; N, 7.0%.

Seeding with D-erythritol 4-phosphate cyclohexylammonium salt, m.p. 183–186° C (20), did not afford a higher melting product. However, after one recrystallization under the same conditions the authentic specimen melted at 138–143° C, and did not depress the melting point of the material having m.p. 133–140° C. The X-ray diffraction powder diagrams of these two low-melting salts were indistinguishable.

In an oxidation in the Warburg respirometer with lead tetraacetate and potassium acetate (21), the erythritol 4-phosphate was observed to consume 2.0 moles of lead tetraacetate (corrected for that used in oxidation of formic acid) and to produce 0.9 moles of formic acid (as determined by the carbon dioxide evolved). These are the values to be expected of a 4-substituted erythritol, whereas for a 3-substituted erythritol 1.0 moles of lead tetraacetate would be used and formic acid would not be produced. This verified that the phosphate group had not migrated.

Oxidation of F-6-P

D-Glyceraldehyde has been prepared readily by oxidation of D-fructose with 2 moles of lead tetraacetate (4) and, by analogy, it appeared likely

that oxidation of F-6-P with 2 moles of lead tetraacetate would yield G-3-P. A solution of potassium F-6-P was prepared from barium F-6-P by the method described above for potassium G-6-P, and the amount of F-6-P present was determined enzymatically. Preliminary experiments showed that at 0° C F-6-P consumed 2 moles of lead tetraacetate very rapidly and a slow over-oxidation then ensued (Table I). To ensure a homogeneous oxidation mixture, it was again necessary to conduct the reaction at low concentrations (1 millimole per liter).

Conditions used for the oxidation of F-6-P with lead tetraacetate were essentially as for the oxidation of G-6-P. The method of recovery of the product differed as follows: at the end of the oxidation period the solution was treated with the calculated volume of 4 *N* sulphuric acid to precipitate all the lead as sulphate, which was filtered off after standing 12 hours at 0° C. The filtrate was concentrated by freeze-drying to 20 ml and treated twice with 50 ml water followed by freeze-drying to 20 ml. The solution was acidified to pH 1 with sulphuric acid, and heated for 10 hours at 50° C to promote hydrolysis of glycollic ester groups (4). After cooling, a 10% excess of barium acetate solution was added and the barium sulphate removed. The filtrate was passed down a column of Amberlite IR-120 (H form) (3 g, air-dried) which was then washed with 100 ml of water. The eluate and washings were adjusted to pH 5 with a solution of 1 *M* ammonium bicarbonate, and concentrated by freeze-drying to 20 ml.

The product from the oxidation of potassium F-6-P (252 μM) with lead tetraacetate (503 μM) was shown by enzymatic assay to contain 123 μM of G-3-P (49% yield). F-6-P and E-4-P were present only in traces, but there was also present 46 μM of other hydrolyzable phosphate (which might have been glycolaldehyde phosphate).

The possibility of producing E-4-P by oxidizing 1 mole of F-6-P with 1 mole of lead tetraacetate was also examined. The oxidation of potassium F-6-P (252 μM) with lead tetraacetate (252 μM) afforded 110 μM of E-4-P (43% yield) together with 24 μM of G-3-P and 20 μM of unchanged F-6-P, as shown by enzymatic assay. This procedure provides yet another method for obtaining E-4-P.

Stability of E-4-P

The stability of E-4-P at various pH values was tested by enzymatic assay after a solution containing 23 μM of E-4-P, 3 μM of G-3-P, and 100 μM of buffer per ml was held at the desired pH for 1 hour at 37° C. The initial pH of the buffers was adjusted in preliminary trials so as to give the desired pH on mixture with the solution of sugar phosphate. After incubation, the tubes containing the mixture were plunged into ice and an equal volume of cold citric acid (100 μM/ml) was added to those at pH 7 or above, cold distilled water to those at pH 3 and 5, and sodium citrate (100 μM/ml) to those in 0.1 *N* hydrochloric acid.

There was no measurable destruction in 0.1 *N* HCl or in buffers at pH 3 or 5. A loss of 15% was observed when a solution of E-4-P at pH 3 was

TABLE II

Percentage of E-4-P and G-3-P destroyed when held at 37° C for 1 hour

Compound	Buffer	pH		
		7.0	8.0	9.0
E-4-P	Versene	2	—	—
	Triethanolamine	5	8	15
	Citrate	3	5	10
	Phosphate	6	—	—
G-3-P	Versene	5	—	—
	Triethanolamine	5	16	22
	Citrate	3	7	16
	Phosphate	19	—	—

stored at −20° C for 1 month. At pH 7 or above E-4-P was slightly more stable than G-3-P (Table II). Erratic results (destruction at pH 8 varied from 30 to 80%) were obtained with glycylglycine buffer. Borenfreund and Dische (22) observed that chelating agents protect phosphoketopentoses and they may likewise protect the phosphoaldoses. When held in 0.1 *N* sodium hydroxide for 1 hour at 37° C, both E-4-P and G-3-P were almost completely destroyed. Destruction during 3 hours at pH 9 in triethanolamine (0.1 *M*) – versene (0.01 *M*) buffer was accompanied by a release of inorganic phosphate, but the total erythrose, as determined by the method of Dische (14) remained constant.

Assay of Transketolase

The crude preparation of E-4-P, though contaminated with G-3-P, was used satisfactorily as substrate for the determination of transketolase (Fig. 2).

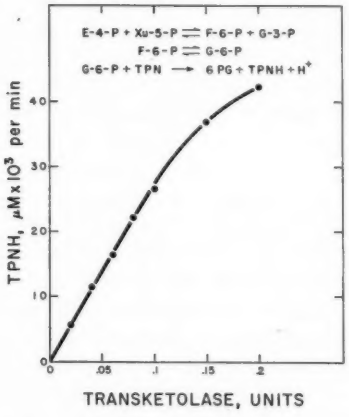


FIG. 2. The relation between reduction of TPN and concentration of transketolase (units as determined by the method of Horecker and Smyrniotis (23)). The reaction mixture consisted of, per milliliter, 20 μM of triethanolamine buffer pH 7.6, 0.25 μM of TPN, 0.6 μM of E-4-P, 0.6 μM of Xu-5-P, 0.1 mg of hexose isomerase, and 1 unit of G-6-P dehydrogenase.

With this system the change in optical density followed a sigmoid curve in which a period of acceleration was followed by a steady linear rate. When the latter rate was corrected for the blank, and plotted as micromoles of TPN reduced per minute against units of transketolase, the relation shown in Fig. 2 was obtained.

Assay of Transaldolase

The proportion of G-3-P in the E-4-P preparation was too high for use in the determination of transaldolase. Accordingly, G-3-P was removed by oxidation with G-3-P dehydrogenase in the presence of 0.1 μM of DPN. The amount of enzyme required to complete the oxidation in an hour was calculated from the rate of oxidation of D-fructose 1,6-diphosphate in the presence of an excess of aldolase. An amount of DPNH oxidase corresponding to twice the above activity also was added. Instead of the oxidase, triosephosphate isomerase and α-GP dehydrogenase may serve to reoxidize the reduced DPN formed by G-3-P dehydrogenase. After the alloted time the pH of the mixture was lowered to 2 with hydrobromic acid and the proteins were removed by centrifugation and rinsed twice. The nucleotides in the combined supernatants were then adsorbed on acid-washed Norit (24). The PGA was removed as the water-insoluble barium salt after neutralization to pH 6.5 and addition of barium acetate. Separation of the E-4-P and PGA on a Dowex-1 formate column may be preferable (19). The E-4-P was then recovered as the barium salt by precipitation with five volumes of ethanol. The losses were large, from 25 to 50%, but the product contained only traces of G-3-P.

The resulting preparation of E-4-P was an excellent substrate for the assay of transaldolase. As seen in Fig. 3, the rate of oxidation of DPNH after subtraction of a small blank was proportional to the concentration of transaldolase, up to 0.02 units as compared with 0.003 units by the method of Horecker and Smyriotis (10).

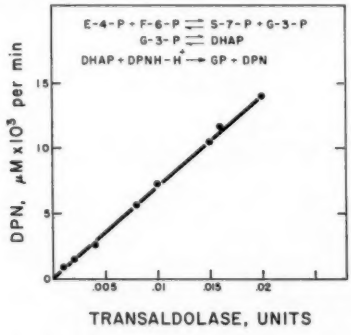


FIG. 3. The relation between the oxidation of DPNH and the concentration of transaldolase (units determined by the method of Horecker and Smyriotis (10)). The reaction mixture contained, per milliliter, 20 μM of triethanolamine buffer pH 7.6, 2 μM of versene, 0.6 μM F-6-P, 0.6 μM E-4-P, 0.09 μM DPNH, and 1 mg of the α-GP dehydrogenase preparation.

Discussion

Although the oxidation of glucose by lead tetraacetate is unaccompanied by over-oxidation, it was not found possible to eliminate over-oxidation completely in the oxidation of G-6-P. Presumably the ionized phosphate group itself acts as a catalyst for over-oxidation as indicated by the deleterious effect of added phosphoric or sulphuric acid on the reaction with D-glucose (Table I). Instability of formate groups in the presence of a carboxylic group was observed earlier in the oxidation of uronic acids with lead tetraacetate (16), and has been demonstrated recently by periodate oxidation (25). For the preparation of pure E-4-P the method of Ballou *et al.* (3) remains the best. The present procedure may be preferred on the merits of convenience and simplicity where absolute purity is not required. The E-4-P preparation contaminated with G-3-P was satisfactory as a substrate for the assay of transketolase and, after removal of G-3-P by enzymatic oxidation, as a substrate for transaldolase.

D-Erythritol 4-phosphate has been isolated from *Propionibacterium pentosaceum* (26) and has been synthesized from D-erythrose (20). The present work provides a convenient two-step synthesis from G-6-P.

G-3-P has been synthesized (27), and can be obtained enzymatically in several ways. Klybas *et al.* (17) have obtained G-3-P and glycolaldehyde phosphate by oxidation of R-5-P with lead tetraacetate. Preparation of G-3-P from F-6-P may provide a useful alternative method.

Acknowledgments

The authors are indebted to Dr. B. K. Bhuyan for the preparation of G-6-P dehydrogenase and to Dr. C. E. Ballou and Dr. H. O. L. Fischer for the gift of authentic samples of D-erythritol 4-phosphate cyclohexylammonium salt and E-4-P dimethyl acetal cyclohexylammonium salt. The technical assistance of Mr. A. S. Sieben and Mrs. Carol Teed is gratefully acknowledged. Thanks are due to Mr. J. A. Baignée for microanalyses and to Mr. T. M. Mallard for X-ray diffraction measurements.

References

1. KORNBERG, H. L. and RACKER, E. Biochem. J. **61**, Proceedings iii (1955).
2. SRINIVASAN, P. R. and SPRINSON, D. B. Federation Proc. **17**, 1245 (1958).
3. BALLOU, C. E., FISCHER, H. O. L., and MACDONALD, D. L. J. Am. Chem. Soc. **77**, 5967 (1955).
4. PERLIN, A. S. and BRICE, C. Can. J. Chem. **33**, 1216 (1955).
5. HORECKER, B. L., SMYRNIOTIS, P. Z., and KLENOW, H. J. Biol. Chem. **205**, 661 (1953).
6. SIMPSON, F. J. Unpublished procedure.
7. CORI, G. T., SLEIN, M. W., and CORI, C. F. J. Biol. Chem. **173**, 605 (1948).
8. RACKER, E. J. J. Biol. Chem. **196**, 347 (1952).
9. KORNBERG, A. and HORECKER, B. L. *In* Methods in enzymology. *Edited by* S. P. Colowick and N. O. Kaplan. Vol. 1. Academic Press, Inc., New York. 1955. p. 323.
10. HORECKER, B. L. and Smyrniotis, P. Z. *In* Methods in enzymology. *Edited by* S. P. Colowick and N. O. Kaplan. Vol. 1. Academic Press, Inc., New York. 1955. p. 381.
11. HOCHSTER, R. M. and KATZNELSON, H. Can. J. Biochem. and Physiol. **36**, 669 (1958).
12. CORDNER, J. P. and PAUSACKER, K. H. J. Chem. Soc. 102 (1953).

13. FISKE, C. H. and SUBBAROW, Y. J. Biol. Chem. **66**, 375 (1925).
14. DISCHE, Z. and DISCHE, M. R. Biochim. et Biophys. Acta, **27**, 184 (1958).
15. COOPER, J., SRERE, P. A., TABACHNICK, M., and RACKER, E. Arch. Biochem. Biophys. **74**, 306 (1958).
16. GORIN, P. A. J. and PERLIN, A. S. Can. J. Chem. **34**, 693 (1956).
17. KLYBAS, V., SCHRAMM, M., and RACKER, E. In press.
18. KLEVSTRAND, R. and NORDAL, A. Acta Chem. Scand. **4**, 1320 (1950).
19. SCHRAMM, M. Private communication.
20. MACDONALD, D. L., FISCHER, H. O. L., and BALLOU, C. E. J. Am. Chem. Soc. **78**, 3720 (1956).
21. PERLIN, A. S. J. Am. Chem. Soc. **76**, 5505 (1954).
22. BORENFREUND, E. and DISCHE, Z. Biochim. et Biophys. Acta, **25**, 215 (1957).
23. HORECKER, B. L. and SMYRNIOTIS, P. Z. *In* Methods in enzymology. *Edited by* S. P. Colowick and N. O. Kaplan. Vol. 1. Academic Press, Inc., New York. 1955. p. 371.
24. CRANE, R. K. and SOLS, A. *In* Methods in enzymology. *Edited by* S. P. Colowick and N. O. Kaplan. Vol. 1. Academic Press, Inc., New York. 1955. p. 277.
25. HOUGH, L., TAYLOR, T. J., THOMAS, G. H. S., and WOODS, B. M. J. Chem. Soc. 1212 (1958).
26. SHETTER, J. K. J. Am. Chem. Soc. **78**, 3722 (1956).
27. BALLOU, C. E. and FISCHER, H. O. L. J. Am. Chem. Soc. **77**, 3329 (1955).

THE EFFECT OF PREVIOUS EXPOSURE TO COLD ON SHOCK SECONDARY TO LIMB ISCHAEMIA[1]

H. SCHACHTER, S. SIDLOFSKY, D. G. BAKER,
J. R. HAMILTON, AND R. E. HAIST

Abstract

Previous exposure to a cold environment for a period of 12 weeks increased the incidence of survival and prolonged the survival time of heavy rats shocked by a clamping technique. The oxygen consumption at 27° C in rats previously exposed to cold for 12 weeks was elevated. During the clamping period the oxygen consumption became more nearly the same in the cold-acclimatized and control rats. After release of the clamps there was a slower fall in oxygen consumption in the previously cold-acclimatized rats than in the control animals. The body temperature, measured in the colon, fell more slowly in the previously cold-exposed rats than in the control rats. This slower fall in colonic temperature was found also in rats previously exposed to cold for shorter periods and was not necessarily associated with changes in survival time. Rats exposed to cold for 6 weeks were "acclimatized" in the sense that they withstood exposure to cold after removal of their fur, but they failed to show significantly lengthened survival times when shocked by this clamping procedure. The administration of thyroxine *shortened* survival times in the shocked rats but reduced the rate of fall of colonic temperature. The administration of cortisone, thyroxine, and cortisone plus thyroxine shortened survival times in the shocked rats.

In connection with studies concerning the influence of environment on shock secondary to limb clamping in rats it was found that previous exposure to a cold environment prolonged the survival times and reduced the rate of fall of body temperature and of metabolic rate. The details of these studies will be presented here.

Materials and Methods

Female rats of the Wistar strain (Carworth Farms) were used in all experiments. They were suspended in adhesive tape slings and both hind limbs were clamped with broad metal clamps under sufficient pressure to stop the circulation, for a period of 10 hours, according to the procedure of Haist and Hamilton (1). The clamps were then removed. The circulation to the previously ischaemic extremities thus being re-established, the limbs became red and swollen and the rats died within a few hours. The rats were kept in a constant-temperature box at 27° C. Food and water were withheld until 24 hours after clamp removal, at which time water alone was given. Rats surviving for 48 hours after clamp release were counted as survivals. Our experience has been that such rats will live indefinitely unless their legs become gangrenous.

Survival times after clamp release were measured by determining the time when the heart stopped beating. Safety-pin electrodes applied to the axillary skin folds picked up the change in electrical potential with each heartbeat. This was amplified and recorded on an Esterline Angus Operation recorder.

[1]Manuscript received August 12, 1958.
Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

A special switching device operated by a synchronous motor allowed records to be obtained in succession from 10 to 12 rats (2).

The colonic temperatures were recorded, at intervals, using thermocouples at a constant depth of insertion (50 mm).

The rates of oxygen consumption were measured before and during shock in several experiments. A closed-circuit apparatus described by Ferguson and Sellers (3) was used and the oxygen consumption of separate rats for a 30-minute period at 27° C was measured.

For the cold exposure, the rats were kept in individual metal cages in a cold room at 1.5° C. They were given Master Feeds Fox Breeder Ration and water ad libitum. The control animals were from the same shipment of rats but were kept at room temperature and given the same diet and general care.

The animals pretreated with thyroxine were given sodium thyroxine (BDH) dissolved in 0.5 *N* NaOH solution and neutralized with 1 *N* HCl just prior to its subcutaneous injection. Control rats were given equivalent amounts of the neutralized solution but no thyroxine.

Experiments and Results

The Effects of Previous Cold Exposure on Subsequent Shock Due to Clamping

Rats were kept in the cold room at 1.5° C as described above for periods of 70 hours to 12 weeks. Other rats from the same shipment were kept at room temperature on a similar regime. The rats were then clamped and shocked as outlined under Methods. Estimations were made of survival times, colonic temperatures, and oxygen consumption after clamp release.

The observations concerning survival times are summarized in Table I. The rats were arbitrarily divided into two weight groups; group I included all rats under 220 g at the time of clamping and group II all rats over 220 g at the time of clamping. Some rats survived for longer than 48 hours. These were excluded from the calculation of survival times. *t*-Tests for differences in survival times between the test and control rats show that rats of weight group II, exposed to cold for 12 weeks prior to clamping, have a significantly lengthened survival time compared to controls of the same age kept at room temperature for the same period. The incidence of survival for 48 hours is greater also. The percentage survival plotted against time for rats in experiments CD and CDH are shown in Fig. 1. From Table I it is evident that in experiments CD, CDH, and CDR, the control rats of the same age as the test rats were heavier at the time of clamping. The changes in body weight in control and test rats are given in Fig. 2 for experiment CDH. Rats exposed to cold for 12 weeks were put in the cold room at time A. Rats exposed to cold for 6 weeks were put in the cold at time B. Rats were clamped at time C. It is interesting to note that the rats exposed to cold for 6 weeks had a considerably higher body weight than the rats of the 12-week group at the time they were put in the cold room, but their body weights just prior to clamping were practically the same as those of the rats exposed for 12 weeks. Weight controls for the cold-exposed rats

TABLE I

Survival times of rats previously exposed to cold

Expt.	No. of rats	Period of cold exposure at 1.5° C	Weight group*	Mean wt. before clamping, g	Weight range before clamping, g	Mean survival time (S.T.) in hours (excluding surviving rats)	Survivals, >48 hr	*t*-Test for differences in S.T. between test group and age controls
CD	20	Age controls	II	261	232–300	9.1±1.0	0	—
	22	Weight controls	I, II	230	192–300	7.6±0.9	0	$P>0.10$
	12	12 weeks	I	205	191–219	12.0±1.9	0	$P>0.10$
	12	12 weeks	II	250	232–265	16.3±1.7	2	$P<0.01$
	24	12 weeks	I, II	226	191–265	14.0±1.3	2	$P<0.01$
CDST	8	Age controls	I, II	221	206–232	5.4±1.1	0	—
	10	70 hours	I, II	230	206–258	6.9±1.2	0	$P>0.10$
CDH	32	Age controls	II	270	235–319	9.2±1.0	1	—
	23	6 weeks	II†	251	215–315	11.3±1.8	2	$P>0.10$
	31	12 weeks	II†	252	210–295	15.1±1.5	4	$P<0.01$
CDR	8	Age controls	I, II	227	205–244	7.9±1.3	0	—
	10	3 weeks	I	205	194–214	8.6±1.7	0	$P>0.10$
	8	6 weeks	I†	204	185–232	7.0±1.5	0	$P>0.10$

NOTE: Mean survival times in hours after clamp release are followed by standard errors. Rats surviving 48 hours are *excluded* from survival time calculations.
*Weight group I, <220 g; weight group II, 220 g and over.
†Most rats were in this weight group.

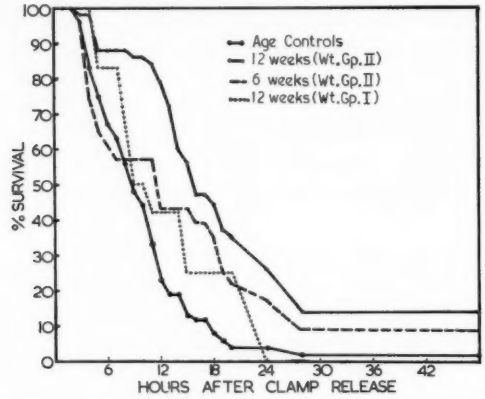


FIG. 1. Percentage of rats surviving at given times after clamp release.

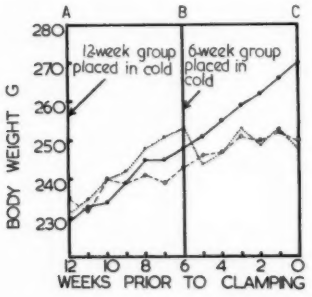


FIG. 2. The changes in mean body weight with time in control rats at room temperature and in rats placed in the cold room 12 weeks prior to clamping and 6 weeks prior to clamping. ●——● Age controls, C- - - -○ 12 weeks, ... 6 weeks.

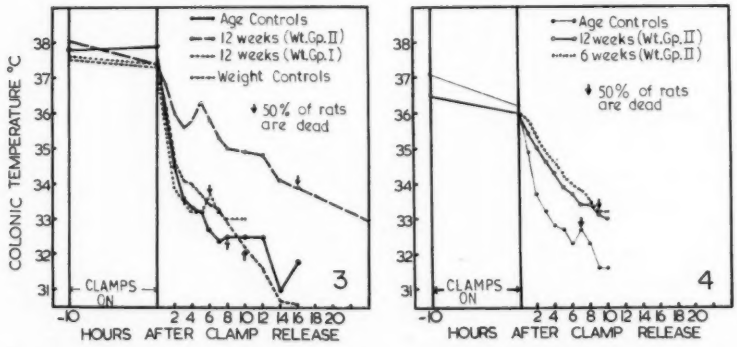


FIG. 3. Mean body temperatures before and after clamp release in rats previously exposed to cold for 12 weeks and in control animals.

FIG. 4. Mean body temperatures before and after clamp release in rats previously exposed to cold for 12 weeks and for 6 weeks and in control animals.

were used in experiment CD but were found to be unnecessary and were omitted from subsequent experiments. Although weight controls are lighter than age controls, the results indicate that the survival times between these two groups do not differ significantly.

The changes in colonic temperatures for experiments CD, CDH, CDR, and CDST are summarized in Figs. 3, 4, 5, 6 respectively. These show mean colonic temperatures of surviving rats. These charts indicate that exposure to cold for a 3-week period or longer, and possibly even for 70 hours, leads to a slower rate of fall of colonic temperature following clamp release. This effect seems to be unrelated to weight groups and survival times.

Studies of the changes in metabolic rates were made on a separate group of rats. Seventeen rats were exposed to cold at 1.5° C for 12 weeks and 15 control rats were used (age and weight controls). The mean weight of the cold-exposed rats at the time of clamping was 214 g. The rats were shocked at 27° C by the clamping technique described previously. Oxygen consumption

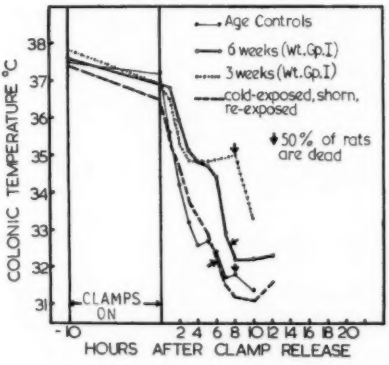


FIG. 5. Mean body temperature before and after clamp release in rats previously exposed to cold for 3 weeks and 6 weeks and in rats previously cold-exposed for 6 weeks and then shorn and re-exposed.

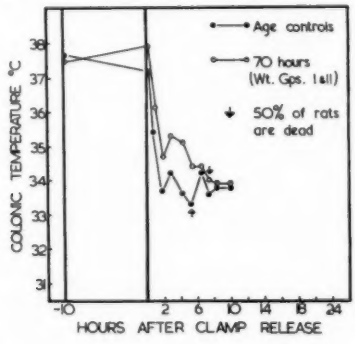


FIG. 6. Mean body temperatures before and after clamp release in rats exposed to cold for 70 hours.

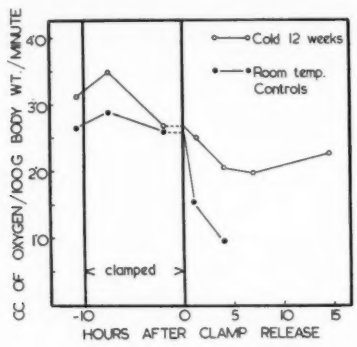


FIG. 7. Mean oxygen consumption before and after clamp application and release in rats (weight groups I and II) previously exposed to cold for 12 weeks and in control animals.

measurements at 27° C were made at times before and after clamp release. The results are plotted in Fig. 7. It can be seen that the rats previously exposed to cold for 12 weeks showed a slower fall in oxygen consumption after clamp release than did the control animals.

The results of this first series of experiments would indicate that, with heavy rats, shocked by a clamping technique, a prior period of 12 weeks' exposure to a cold environment (1.5° C) leads to increased incidence of survival, a prolongation of survival time, a slower reduction in colonic temperature, and a slower fall in oxygen consumption.

The Relationship between "Acclimatization to Cold" and Survival Times in Shock Secondary to Limb Clamping

The ability of rats to survive exposure to cold at 1.5° C, when their fur is removed by clipping, has been used as one criterion of "acclimatization" to cold (4, 5). Using this criterion it was reported that acclimatization to cold is maximal after 4 to 6 weeks' exposure (6). Since there were differences in our experiments between the rats exposed to cold for 6 weeks and those exposed for 12 weeks it seemed necessary to test whether or not our rats exposed for 6 weeks were "cold-acclimatized".

Rats were kept in the cold for 4 to 5 weeks. They were then anaesthetized with ether and clipped at room temperature. A few hours were allowed for recovery and then the animals were returned to the cold for an additional week. All 10 rats survived and were then shocked at 27° C by the method previously described. Eight control rats of the same age were kept at room temperature. After the release of the clamps colonic temperatures and survival times were recorded. Oxygen consumption at 27° C was measured prior to clamping. The adrenal glands were weighed at the time of death. This experiment formed part of a larger experiment (CDR in Table I).

The results of this experiment are summarized in Table II and Fig. 5. The results indicate that these animals can withstand a cold environment

TABLE II

Relationship between cold acclimatization and survival time in shock secondary to limb clamping

Expt. CDR, group	No. of rats	Mean body weight prior to clamping, g	Mean O_2 consumption prior to clamping	Mean adrenal weights at time of death: mg	Mean adrenal weights at time of death: mg/g final weight	Mean survival time
Age controls	8	227	113±4.1	51.4	0.25	7.9±1.3
Cold exposure, 3 weeks	10	205	169±6.4	58.0	0.33	8.6±1.7
Cold exposure, 6 weeks	8	204	160±8.8	63.0	0.36	7.0±1.5
Cold exposure, 5 weeks, clipped, exposed to cold for another week	10	196	199±9.2	66.9	0.40	8.9±1.7

NOTE: Experiment CDR of Table I and "clipped" group. Survival times in hours after clamp release are followed by standard errors. Mean oxygen consumption at 27° C was measured prior to clamping and is expressed in cc of oxygen at N.T.P. per min per m^2 of body surface area, followed by standard error. Surface area in $cm^2 = 12.54W^{2/3}$, where W = body weight in grams.

TABLE III

Effect of pretreatment with thyroxine on survival time

Thyroxine dosage	Experimental group	No. of rats	Mean body weight prior to clamping, g	Mean O_2 consumption prior to clamping	Mean survival time (S.T.) in hours	t-Test for differences in S.T. between test group and controls
16 mg/kg	Controls	23	218	137	7.8±1.2	—
	Thyroxine pretreated	23	211	205	3.0±0.4	$P<0.01$
12 mg/kg	Controls	21	230	131	6.1±1.0	—
	Thyroxine pretreated	18	222	185	4.4±0.7	$P>0.10$

NOTE: Mean survival times in hours after clamp release are followed by standard errors. Mean oxygen consumption at 27° C was measured prior to clamping and is expressed in cc of oxygen at N.T.P. per min per m^2 body surface area. Surface area in $cm^2 = 12.54W^{2/3}$, where W = body weight in grams.

(1.5° C) even after their fur was removed. Despite this, the survival times are not significantly different from those of the control animals. The "acclimatized", clipped animals had a higher oxygen consumption before clamping but the colonic temperatures fell as rapidly as in the control animals, following clamp release. This may be due to greater heat loss due to lack of insulating fur. The results of this experiment indicate that the changes in the animal causing acclimatization to cold, as shown by survival in the cold following removal of the fur, probably are not the changes leading to the prolongation of survival time in shock following limb ischaemia. In any event, further changes or other changes appear to take place in the rat well beycnd the 4- to 6-week period of cold exposure.

The Effect of Thyroxine Pretreatment on Shock Secondary to Limb Clamping

Rats were injected subcutaneously with thyroxine for a period prior to the production of shock. One group of rats received four subcutaneous injections of thyroxine (4 mg/kg) during an 8-day period prior to clamping. Another group of rats received six daily subcutaneous injections of thyroxine (2 mg/kg) prior to clamping. A third group received a course of injections similar to that of group 2. Survival times and colonic temperatures were followed in the first and second groups. Oxygen consumption measurements were made on the rats of group 3. Control animals, not receiving thyroxine, were used for each of these groups.

The survival times are presented in Table III and the changes in colonic temperature in Figs. 8 and 9. The oxygen consumption results are plotted in Fig. 10.

These results indicate that pretreatment with the higher dose of thyroxine decreased the survival times. Decreasing the dose eliminated the significance of the effect. The pretreatment with thyroxine resulted in the maintenance of

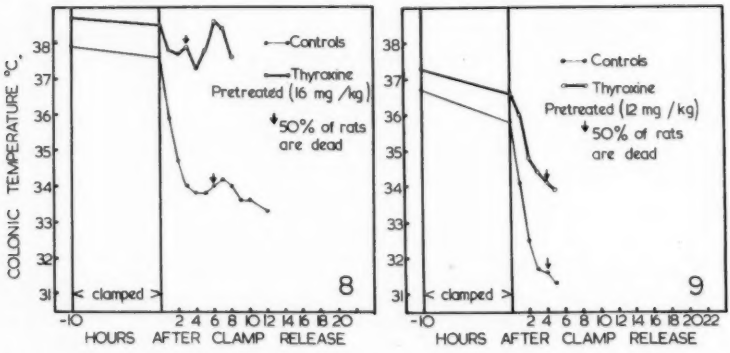


FIG. 8. Mean body temperatures before and after clamp release in rats pretreated with thyroxine (total dose 16 mg/kg) and in non-treated animals.

FIG. 9. Mean body temperatures before and after clamp release in rats pretreated with thyroxine (total dose 12 mg/kg) and in non-treated rats.

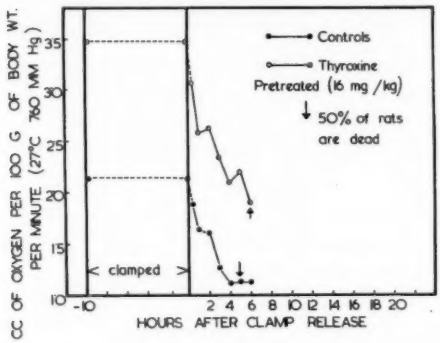


FIG. 10. Mean oxygen consumption before and after clamp release in rats pretreated with thyroxine (16 mg/kg) and in control animals.

a higher colonic temperature following clamp release. The oxygen consumption also was maintained at a higher level in the thyroxine-pretreated group than in control animals, after the release of the clamps. As in the rats exposed to cold for 6 weeks or less, the better maintenance of the colonic temperature after clamp release does not seem to be related to an increased survival time.

Effect of Pretreatment with Cortisone and with Cortisone plus Thyroxine

Previous reports had indicated that "artificial acclimatization" could be achieved by the use of cortisone and thyroxine together (5). An attempt was made to find whether or not this pretreatment might have an effect in the shocked animal similar to that of cold exposure for 12 weeks.

One group of rats received injections of 1 mg cortisone (Merck Cortone Acetate in saline suspension) per rat per day for 7 successive days prior to clamping. A second group of rats received 0.07 mg of thyroxine sodium (BDH) per rat per day for 7 successive days prior to clamping (about 0.3 mg/kg). A third group received 1 mg cortisone plus 0.07 mg thyroxine sodium daily for 7 successive days prior to clamping. A fourth group of control rats received injections of saline in volumes comparable to the fluid injected in the other tests.

Tests for oxygen consumption were made on four thyroxine-pretreated rats and four saline-injected controls just prior to clamping. The thyroxine-pretreated animals showed a mean oxygen consumption of 6.7 cc per minute whereas the saline-injected controls had a mean oxygen consumption of 5.1 cc per minute.

The effects of the different pretreatments on survival are given in Table IV.

It is evident that these pretreatments shortened rather than lengthened survival times in shock secondary to limb clamping, and that the shortening with cortisone plus thyroxine was greater than the shortening with thyroxine alone. Together, these materials lead to a fall in body weight. The colonic temperatures fell somewhat more slowly after clamp release in the thyroxine-pretreated rats with or without cortisone.

TABLE IV

Effect of cortisone and thyroxine pretreatment on survival times

Experimental group	No. of rats	Mean body weight prior to injection, g	Mean body weight prior to clamping, g	Mean survival time (S.T.) in hours	*t*-Test for differences in S.T. between test group and controls
Cortisone	11	225	226	3.8 ± 1.4	$P < 0.05$
Thyroxine	8	223	225	3.4 ± 1.6	$P < 0.05$
Cortisone + thyroxine	16	220	214	2.7 ± 1.5	$P < 0.01$
Saline controls	12	222	231	5.5 ± 2.1	—

NOTE: Mean survival times in hours after clamp release are followed by standard errors.

Discussion

The results of these experiments indicate that exposure to a cold environment for a period of 12 weeks significantly prolongs the survival time in heavy rats shocked by a clamping technique. In lighter rats no significant effect was obtained. Rats exposed to cold for a period of 6 weeks or less did not show significant prolongation of survival time.

The oxygen consumption at 27° C of rats previously exposed to cold for 12 weeks was elevated. After a period of clamping, the oxygen consumption in control and test animals became nearly the same, but following clamp release the fall in oxygen consumption in the previously cold-exposed animals was slower than in the control animals which had been kept at room temperature. Also the rectal temperature did not drop as quickly in the previously cold-exposed animals after clamp release. This less rapid fall in rectal temperature was observed in the animals exposed to short periods in the cold as well as in animals exposed for the longer period and does not seem to be related to the prolongation of survival time. One criterion of "cold acclimatization" is the ability of clipped rats to survive cold exposure at 1.5° C. By this criterion the rats with 4 to 5 weeks' cold exposure were "acclimatized". While these animals were able to withstand the cold environment they did not show prolongation of survival time in shock secondary to limb clamping.

The better survival in the heavy, shocked rats exposed to cold may be due to the increased ability of heavier rats to withstand cold. Other reports indicate that heavier rats resist cold better than lighter ones (4). Immediately after exposure to cold the animals did not show their normal gain in body weight. Later this was resumed. It may be that only after the animal has resumed its increase in body weight in the cold environment is there an evident effect of cold exposure on survival. It has been shown by Sellers and co-workers that there is a gradual increase in metabolic rate (30° C) with 1 to 2 weeks' exposure to cold. This was believed to be due principally to increased visceral metabolic activity rather than to an increased activity of skeletal muscle. There is at the same time a significant increase in the temperature of the colon in cold-exposed rats (7, 8, 9). It is possible that increased visceral metabolic activity may be responsible for the slower drop in metabolic rate and in rectal temperature of cold-exposed rats subjected to limb ischaemia.

Clipped acclimatized rats, on exposure to cold (1.5° C), show a greatly increased ability to *maintain* a high metabolic rate when compared with clipped non-acclimatized controls (5, 6, 9). Whatever is responsible for this ability to maintain increased heat production at the low temperature apparently is not responsible for the increased survival times of the cold-exposed rats in shock resulting from limb clamping.

When animals had their metabolic rate increased by pretreatment with thyroxine they showed a reduced rate of fall of body temperature, after clamp release, which was similar to that found in previously cold-exposed animals.

The survival times in the thyroxine-pretreated rats, however, were shortened rather than lengthened. When the administration of thyroxine was accompanied by the administration of cortisone, the survival times were shorter still. This combination of thyroxine and cortisone was found by others (6) to improve survival on exposure to cold but this pretreatment had no beneficial effect on survival in shock resulting from limb clamping.

Brief reports of this effect of previous cold exposure have been given before (10, 11) and the beneficial effect on survival has been confirmed by others (12, 13). As yet it is not known why the 12-week previously cold acclimatized rats have longer survival times when shocked by a standard clamping procedure and why they show an increased incidence of survival following limb clamping. There are reports which indicate that exposure to cold over a period of time leads to an increase in blood volume (14, 15, 16). Conceivably, this increased blood volume at the time of clamp release is a factor of major importance in promoting survival in these animals. With an equivalent loss of fluid from the circulation there would be a smaller proportion of the total blood lost. It has been reported also that sodium is retained in cold-acclimatized animals (14). These changes in electrolyte balance and in blood volume may be factors of importance in the beneficial effect of prolonged previous exposure to cold on survival in shock following limb clamping. Previous cold acclimatization was reported to cause no alteration in muscle chemistry and temperature during and following limb ischaemia or in the increase in the water of muscle after ischaemia (12). The changes in blood flow to the liver after ischaemia also were not altered, but there was a lesser change in the temperature of the liver in the postischaemic period (13). Other reports indicate that there is, at first, an increase in oxygen consumption of liver in vitro (17), and of the animal as a whole (18), on exposure of an animal to cold, but that this reaches a maximum in 1 month and then declines. There is also a progressive decrease in anaerobic glycolysis in liver tissue in vitro after prolonged exposure to cold (17). As yet, no relation between specific metabolic changes resulting from cold exposure and the beneficial effect of previous cold exposure in the shocked animal has been established. The evidence, however, is sufficient to indicate that the effect of previous cold exposure on energy supply in the *shocked* animal merits further detailed investigation.

Acknowledgment

This work was supported by a grant from the Defence Research Board of Canada.

References

1. HAIST, R. E. and HAMILTON, J. I. J. Physiol. **102**, 471 (1944).
2. SMITH, L. C. and HAIST, R. E. Can. J. Biochem. and Physiol. **33**, 553 (1955).
3. FERGUSON, J. K. W. and SELLERS, E. A. J. Pharmacol. Exptl. Therap. **97**, 177 (1949).
4. SELLERS, E. A., YOU, S. S., and THOMAS, N. Am. J. Physiol. **165**, 481 (1951).
5. SELLERS, E. A., REICHMAN, S., THOMAS, N., and YOU, S. S. Am. J. Physiol. **167**, 651 (1951).
6. SELLERS, E. A., REICHMAN, S., and THOMAS, N. Am. J. Physiol. **167**, 644 (1951).
7. SELLERS, E. A. and YOU, S. S. Am. J. Physiol. **163**, 81 (1950).

8. YOU, R. W. and SELLERS, E. A. Endocrinology, **49**, 374 (1951).
9. SELLERS, E. A., SCOTT, J. W., and THOMAS, N. Am. J. Physiol. **177**, 372 (1954).
10. HAIST, R. E., SCHACHTER, H., SIDLOFSKY, S., HAMILTON, J. R., and BAKER, D. G. Federation Proc. **15**, 86 (1956).
11. HAIST, R. E. Shock and circulatory homeostasis. *Edited by* H. D. Green. Josiah Macy Jr. Foundation, New York. 1957. p. 303.
12. THRELFALL, C. J. and STONER, H. B. Brit. J. Exptl. Pathol. **38**, 339 (1957).
13. STONER, H. B. Brit. J. Exptl. Pathol. **39**, 251 (1958).
14. BAKER, D. G. and SELLERS, E. A. Can. J. Biochem. and Physiol. **35**, 631 (1957).
15. BAUGH, C. W., BIRD, G. S., BROWN, G. M., LENNOX, C. S., and SEMPLE, R. E. J. Physiol. **140**, 347 (1958).
16. DEB, C. and HART, J. S. Can. J. Biochem. and Physiol. **34**, 959 (1956).
17. HANNON, J. P. Am. J. Physiol. **192**, 253 (1958).
18. COTTLE, W. and CARLSON, L. D. Am. J. Physiol. **178**, 305 (1954).

THE DETERMINATION OF CALCIUM AND MAGNESIUM IN BLOOD USING ONE INDICATOR[1]

H. M. Czajkowska Robinson and J. C. Rathbun

Abstract

A rapid ethylenediaminetetraacetic acid titration for calcium and magnesium in serum requiring 0.1 ml of blood is described. Eriochrome black T is used as the sole indicator and the end point is observed photometrically. After determination of total calcium and magnesium, the calcium is removed as oxalate, the magnesium determined separately, and the calcium obtained by difference. The method is highly reproducible and is sensitive to ±0.1 mg% of either calcium or magnesium.

Introduction

Recently published methods for the complexiметric determination of calcium and magnesium describe, almost without exception, the use of EDTA* with two indicators. Of the latter, one is usually specific for calcium (e.g. murexide) while the other may be used with a calcium-free sample to determine magnesium directly (1), or with a "whole" sample to obtain the total of calcium and magnesium and the latter found by difference. Excellent reviews of these methods have been published by Barnard (2) and by Flaschka and co-workers (3).

Because of the instability and cost of murexide and other calcium-specific indicators, as well as the general inconvenience of having to use two different buffered indicator solutions, a method which utilizes one economical, stable indicator would be useful. Such a technique, utilizing eriochrome black T only, has been reported (4) but it is not a titrimetric procedure, does not utilize EDTA, and was not applied to biological samples but rather to macro samples (50 ml) of water. The method described here has proved to be accurate, rapid, and convenient for the microassay of calcium and magnesium in blood as well as in water solutions.

The procedure is based upon the micromethod of Wilkinson (5) but differs in that the calcium is selectively removed from solution with oxalate and the residual magnesium is determined accurately using eriochrome black T and EDTA, buffered at pH 10.4. Using the same indicator on a "whole" sample, the total calcium plus magnesium is determined and the calcium is obtained by difference. The quantitative removal of calcium, in the presence of magnesium, is a development of Clark and Collip's method (6), the process being greatly accelerated by shaking (7).

[1]Manuscript received October 6, 1958.
Contribution from the Paediatric Research Laboratory, War Memorial Children's Hospital, London, Ontario, and the Department of Paediatrics, University of Western Ontario, London, Ontario. This work was made possible by a Federal Health Grant (Child and Maternal Health), administered by the Province of Ontario.

*Ethylenediaminetetraacetic acid, used as a complexing agent.

Methods

1. Apparatus

A Coleman Junior spectrophotometer, model 6A, with matched square cuvettes (13×13×100 mm, No. 14-307) is used at 660 mμ, the end point being the point of minimum per cent transmission.

An "Agla" micrometer syringe–burette (Burroughs Wellcome Ltd.) fitted with a bent glass needle tip. The burette capacity is 0.5 ml, and it is graduated in 0.2 μl units.

Delivery pipettes, Long-Levy type (Microchemical Specialties Inc. No. 284-B).

2. Reagents

All reagents were mixed and stored in polyethylene containers; distilled water was doubly distilled in all-glass apparatus and stored in polyethylene.

Calcium and magnesium standards, EDTA stock solution, absolute methanol, ethanolamine, and the buffered indicator solution were all prepared as described by Wilkinson (5). (See Addendum.)

The EDTA working solution was prepared by diluting 100 ml of the stock solution to 1 liter.

Eriochrome black T solution.—One hundred milligrams of the dye (Pharmaceutical Laboratories, National Aniline Division, New York) was dissolved in 100 ml of absolute methanol and 4 ml of concentrated ammonium hydroxide (density 0.9) was added. This solution proved to be stable for at least six months.

Ammonium oxalate.—Reagent ammonium oxalate monohydrate (2 g) was dissolved in 98 ml of water.

3. Procedure

All glassware is rinsed with, and stored under, doubly distilled water in polyethylene containers. The microburette is rinsed several times with EDTA solution before it is mounted on its stand. The plunger is not lubricated and, when in use, the tip is kept submerged.

Blood collection.—Blood is obtained by heel puncture and allowed to flow freely into glass or polyethylene tubing. The tubes are sealed and centrifuged. The serum is collected by cutting off the tube at the sedimentation border line.

Calcium precipitation.—Into a 13×100 mm test tube, 100 μl of serum and 100 μl of the 2% ammonium oxalate solution are pipetted. The tube is covered with Parafilm (Marathon Corporation) to minimize evaporation, shaken 15 minutes on a Kahn shaker, and centrifuged at 2000 r.p.m. for 5 minutes. Of the supernatant 100 μl are used for the magnesium determination.

Titration.—The spectrophotometer is zeroed at 660 mμ against a distilled water blank. A fresh, matched cuvette containing 5 ml of eriochrome buffer is inserted, and 100 μl of sample (standard or serum) is added with immersion

of the tip of the delivery pipette. The contents are mixed using a Pasteur pipette and the tip of the clamped microburette is introduced beneath the surface. EDTA is added in increments, volume and per cent transmission readings being recorded after each addition.

The solution is well mixed with a Pasteur pipette between each increment, which should be reduced in volume as the end point is approached. The first appearance of color stability, indicated by minimum per cent transmission, is taken as the end point of the titration.

On completion of each titration, the cuvette is rinsed with eriochrome buffer.

4. Calculations

For magnesium,

$$M = (A/B)\ \text{mg\%}$$

where

M = mg of magnesium per 100 ml of sample,

A = {(EDTA units* for the calcium-free sample) − (EDTA units for the water blank)} × 2,

B = (EDTA units for a 1 mg% magnesium standard) − (EDTA units for the water blank).

For calcium,

$$C = \{(D-A)/E\}\ \text{mg\%}$$

where

C = mg of calcium per 100 ml of sample,

D = (EDTA units for a "whole" sample) − (EDTA units for the water blank),

A = as in the magnesium determination,

E = (EDTA units for a 1 mg% calcium standard) − (EDTA units for a water blank).

With practice, B, E, and the water blanks assume constant values for a particular apparatus and operator.

Discussion

Abandonment of the use of a calcium-specific indicator was desirable because of unsatisfactory results with murexide, calcon, and calcein. This became possible with the development of a successful technique for the removal of calcium from the serum quantitatively, specifically, and rapidly. Then it was practicable to make use of eriochrome black T for both calcium plus magnesium, and magnesium alone. That the calcium removal is satisfactory is made evident by the data given in Table I. The data of Table II show that the magnesium content of standard solutions is not affected by the calcium-removal technique. In Table III the data indicate that there is no significant coprecipitation of magnesium with the calcium oxalate, while Table IV shows the calcium and magnesium content of normal blood serum, as well as the recovery of added calcium and magnesium.

*One EDTA unit is equivalent to one division on the micrometer scale of the Agla burette.

TABLE I

Calcium found (mg%) in standard aqueous solutions of calcium chloride before and after treatment with ammonium oxalate with 15 minutes' shaking

5 mg% Ca		10 mg% Ca		20 mg% Ca	
Before	After	Before	After	Before	After
5.1	0	10.0	0	20.1	0
5.1	0.12	10.2	0	20.0	0.12
4.9	0	10.0	0	20.0	0
5.0	0.12	10.0	0.12	19.9	0

TABLE II

Magnesium found (mg%) in standard aqueous solutions of magnesium sulphate before and after subjection to the calcium precipitation technique

1 mg% Mg		2 mg% Mg		2.78 mg% Mg	
Before	After	Before	After	Before	After
1.02	1.02	2.00	2.06	2.70	2.86
1.09	0.91	2.00	2.06	2.70	2.62
1.14	1.02	2.00	2.16	2.80	2.74
1.02	1.02	1.94	2.06	2.74	2.86

TABLE III

Magnesium found (mg%) in standard solutions containing chloride and magnesium sulphate after subjection to calcium precipitation

5 mg% Ca plus:		10 mg% Ca plus:		
1 mg% Mg	2 mg% Mg	1 mg% Mg	2 mg% Mg	2.78 mg% Mg
1.1	2.0	1.1	2.0	3.0
1.1	2.0	1.1	2.0	3.0
1.0	2.0	1.1	2.0	2.67
1.1	2.0	1.1	2.0	2.78

TABLE IV

Calcium and magnesium found (mg%) in normal human serum and in serum containing added calcium and magnesium

Normal serum		Normal serum plus 10 mg% Ca		Normal serum plus 2 mg% Mg	
Ca	Mg	Ca	Mg	Ca	Mg
9.7	2.0	20.0	2.0	9.6	4.0
9.7	2.0	19.7	2.0	9.6	4.0
9.7	2.0	19.7	2.0	9.6	4.0
9.7	2.0	19.7	2.0	9.6	4.0

The data are only a few representative figures taken from over seven hundred titrations using standards and serum. The entire procedure for calcium and magnesium, in duplicate, requires 25 to 30 minutes from the time of serum separation; it demonstrates a high order of reproducibility as indicated by the data of the tables, and is sensitive to ± 0.1 mg% of either calcium or magnesium. The end point is easily detected and the titration may be interrupted, if necessary, for as much as a half hour without affecting the result. After reaching the end point, the transmittance is stable for at least thirty minutes.

References

1. GEHRKE, G. W., AFFSPRUNG, H. E., and LEE, H. E. Anal. Chem. **26**, 1944 (1954).
2. BARNARD, A. J., BROAD, W. C., and FLASCHKA, H. Chemist Analyst, **45**, 86, 111 (1956); **46**, 18, 46, 76 (1957).
3. FLASCHKA, H., BARNARD, A. J., and BROAD, W. C. Chemist Analyst, **46**, 106 (1957); **47**, 22, 52 (1958).
4. YOUNG, A., SWEET, T. R., and BAKER, B. B. Anal. Chem. **27**, 356 (1955).
5. WILKINSON, R. H. J. Clin. Pathol. **10**, 126 (1957).
6. CLARK, E. P. and COLLIP, J. B. J. Biol. Chem. **63**, 461 (1925).
7. CAMPBELL, W. R. Can. J. Biochem. and Physiol. **35**, 1033 (1957).

Addendum

Chemicals as described by Wilkinson:

Calcium standard (neutral, 10 mg per 100 ml).—Pure dry calcium carbonate (A.R.) 0.250 g was dissolved in 6 ml of *N* hydrochloric acid and heated in a boiling-water bath until the excess of hydrochloric acid was driven off. When cool, the calcium chloride was transferred with washings to a 1-liter flask and diluted to the mark with distilled water.

Stock magnesium standard (100 mg per 100 ml).—Dry $MgSO_4 . 7H_2O$ (A.R.) 10.131 g was dissolved in distilled water and volume made up to 1 liter.

Magnesium standard (2 mg per 100 ml).—Two milliliters of stock standard were diluted to 100 ml with distilled water.

Absolute methanol.—Methanol absolute was redistilled in an all-glass apparatus.

Ethanolamine.—2-Hydroxyethylamine (b.p. 173° C) was redistilled in the same way as the methanol.

EDTA stock solution (4.804 g per liter).—The disodium salt of ethylenediaminetetraacetic acid ($C_{10}H_{14}O_8N_2Na_2 . 2H_2O$) (mol. wt. 372.3) (Eastman Organic Chemicals) was dried at 110° C for 4 hours and placed in a desiccator overnight and then 4.804 g of the salt was dissolved and made up to 1 liter in distilled water.

Buffer indicator solution.—To 100 ml of distilled water were added 1.0 ml of ethanolamine and 1.7 ml of the eriochrome black T stock solution. The buffer indicator with the addition of 0.1 ml of aqueous standard or serum had a pH of 10.4.

ÉTUDE DE LA RÉACTION DE L'ADRÉNALINE AVEC L'ÉTHYLÈNEDIAMINE[1]

GUY NADEAU ET LOUIS-PHILIPPE JOLY

Abstract

The influence of pH, reagent composition, time, temperature, light, and various ions, on the reaction of fluorescence between adrenaline and ethylenediamine, has been investigated. The necessity of a preliminary oxidation to adrenochrome is confirmed. Optimum pH of reaction is situated at 10.4 ± 0.1, while extraction with isobutanol is best performed at pH 10.1 ± 0.1. A satisfactory reagent is one that contains 114.5 millimoles of ethylenediamine base per 100 ml of reacting mixture and sufficient hydrochloric acid to bring the pH to 10.3 ± 0.1. Temperature and length of reaction may be conveniently set at 50° C and 20 to 30 minutes. Increasing the temperature significantly lowers the yield of fluorescent material; performing the reaction at room temperature increases the yield, but lengthens the procedure. Exposure to diffuse and ultraviolet light also affects the yield of fluorescence. All these factors (temperature, light, as well as excess of oxygen) appear to exert their influence in the first stages of the reaction, since the fluorescent material, once formed, is relatively stable. The beneficial effect of various ions, including Cu^{++}, Al^{+++}, and MoO_4^{--}, is discussed in relation to complex formation. Efficiency of extraction with aliphatic alcohols has also been investigated; true yield from C^{14}-adrenaline is estimated. In the light of recent findings on the structure of adrenochrome, new pathways for the reaction are suggested.

Introduction

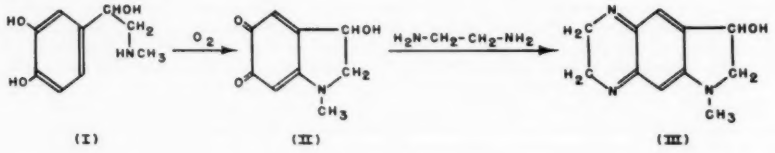
En 1949, Natelson et ses collaborateurs (1) ont proposé, comme méthode de dosage de l'adrénaline, sa réaction avec les diamines (v.g. éthylènediamine) pour former un produit fluorescent soi-disant plus stable que celui que l'on obtient par simple alcalinisation. La réaction procéderait en deux étapes: une oxydation de l'adrénaline en quinone correspondante, suivie d'une condensation avec la diamine. Les auteurs ont, en outre, noté que le catéchol donne une réaction analogue. De plus, selon eux, le matériel fluorescent pourrait s'extraire quantitativement par un alcool aliphatique.

La réaction a été reprise par Weil-Malherbe et Bone (2), qui l'ont appliquée au dosage des catécholamines dans le sang. Les mêmes auteurs ont, peu après, amélioré leur méthode de façon à permettre le dosage différentiel de l'adrénaline et de la noradrénaline (3). Depuis, la technique a été généralement adoptée sans modifications majeures (4, 5, 6, 7). Quelques auteurs ont cependant trouvé que la méthode ne pouvait s'appliquer au dosage des catécholamines du plasma soit à cause de son manque de spécificité, soit à cause de sa sensibilité insuffisante (8, 9); d'autres l'ont toutefois jugée convenable pour des déterminations dans l'urine (10, 11).

[1]Manuscrit reçu le 30 septembre 1958.
Contribution du Département de Chimie, Université Laval, Québec, Qué. et du Laboratoire de Biochimie, Hôpital Saint-Michel Archange, Québec, Qué. Extrait de la thèse soumise par l'un des auteurs (L.-P. J.) à l'École des Gradués de l'Université Laval pour l'obtention du grade de Docteur ès Sciences.

À notre connaissance, personne encore n'a paru mettre en doute les étapes suggérées par Natelson *et al.* (1). Il semble donc admis que la technique, aussi bien que ses nombreuses modifications, repose, du moins implicitement, sur les hypothèses suivantes:

(*a*) Le composé fluorescent (III) s'obtiendrait par condensation de l'éthylènediamine avec la quinone (II) résultant de l'oxydation de l'adrénaline (I) d'après le schéma suivant (2):



(I) (II) (III)

Il n'y aurait donc pas de réaction possible en absence d'air ou d'un agent oxydant. Cette condition a été mentionnée par Weil-Malherbe et Bone et confirmée plus tard par Burn et Field (12). On a supposé un comportement analogue pour la noradrénaline (2).

(*b*) La présence de la diamine sous sa forme ionisée serait essentielle (2), d'où l'emploi d'un mélange d'éthylènediamine et de son chlorhydrate ou encore l'addition d'acide chlorhydrique (13) ou de chlorure d'ammonium (7) à la base.

(*c*) La réaction ne se ferait qu'en milieu alcalin, dans lequel l'adrénaline est auto-oxydable (2). Natelson *et al.* avaient d'ailleurs déjà mentionné que la réaction pouvait se produire en présence d'ammoniaque additionnée d'une amine (éthylènediamine, butylamine, aniline). Burn et Field (12) ont situé le pH optimum de la réaction à 11. Erne et Canbäck (13) ont trouvé que, pour la noradrénaline, le meilleur réactif s'obtenait en mélangeant des volumes égaux de base et d'une solution 2 *M* de son chlorhydrate.

(*d*) L'adrénaline et la noradrénaline ne formeraient respectivement qu'un seul produit fluorescent (1, 2). Toutefois, Burn et Field (12) ont récemment soutenu l'existence d'au moins deux dérivés fluorescents pour chaque catécholamine. Ellman (14) a également signalé l'existence d'un nombre variable de produits suivant les conditions de réaction, sans cependant leur attribuer de propriétés physiques caractéristiques. Par contre, Gray et Young (15) n'ont mis en évidence qu'un seul dérivé pour chaque amine, par chromatographie ascendante sur papier, avec trois systèmes différents de partage. De notre côté, nous avons depuis signalé que la réaction donne lieu à la formation de plusieurs produits séparables par électrophorèse sur papier, ou mieux encore, par chromatographie bidimensionnelle sur papier (16).

(*e*) Dans des conditions appropriées, on pourrait extraire par les alcools aliphatiques tout le matériel fluorescent (1). Weil-Malherbe et Bone (2) ont employé l'alcool isobutylique de préférence à l'alcool *n*-amylique, après avoir saturé la phase aqueuse de chlorure de sodium. Ce mode opératoire permettant une meilleure séparation, au dire des auteurs, a été adopté sans commentaires par la majorité des autres chercheurs.

Parmi les autres aspects de la réaction plus particulièrement étudiés, on note la durée et la température, ainsi que l'influence des catalyseurs ou de la lumière.

Selon la technique originale de Natelson *et al.*, la réaction s'effectue à 100° C durant 1 heure. Bone (17), qui a étudié l'effet de températures (de 15° C à 100° C) et de durées variables (de 5 à 60 minutes), conclut que le maximum de fluorescence peut s'obtenir en chauffant le mélange réactionnel à 50° C durant 20 minutes. Erne et Canbäck (13) ont répété l'expérience pour la noradrénaline et ont adopté des conditions de 5 minutes à 100° C. Kägi *et al.* (7), en modifiant la technique originale, ont spécifié une température de 63° C et un temps de 20 minutes sans fournir cependant de raison précise.

L'action catalytique de certains ions a d'abord été suggérée par Erne et Canbäck (13). Ils ont en effet trouvé que l'addition de molybdate d'ammonium favorise la formation du soi-disant dérivé de la noradrénaline. En fait, l'action catalytique des sels de cuivre et d'autres sels minéraux sur l'oxydation de l'adrénaline était déjà connue (23, 24, 25). C'est pour éviter cette oxydation prématurée que Weil-Malherbe et Bone (2) ont purifié sur échangeur d'ions l'acétate de sodium utilisé au cours de la chromatographie du plasma sur alumine. Ils n'ont cependant pas suggéré de précautions supplémentaires au cours des autres étapes de la technique. De récents travaux, effectués dans notre laboratoire (18), ont montré l'influence de divers ions métalliques sur la fluorescence obtenue au cours de la présente réaction. La présence d'ions Al^{+++} expliquerait, en particulier, l'augmentation de fluorescence observée quand l'adrénaline est dissoute dans de l'acide acétique qui a percolé à travers une colonne d'alumine comparativement à des solutions dans l'acide acétique pur.

Erne et Canbäck (13) ont aussi mentionné une diminution de la fluorescence du dérivé de la noradrénaline par exposition à la lumière ultraviolette. C'est pour la même raison que Aronow et Howard (6) effectuent la réaction en lumière rouge. D'autre part, Valk et Price (8) exposent leurs échantillons à la lumière diffuse pour un temps déterminé, afin d'atténuer et d'uniformiser la diminution de fluorescence du dérivé de la noradrénaline. Ce dernier procédé a été repris par Millar (19). Dans tous les travaux précédemment cités, aucune mention n'est faite de l'influence de la lumière sur la stabilité de la fluorescence du dérivé de l'adrénaline.

Des essais préliminaires effectués dans notre laboratoire (16) ont jeté quelques doutes sur le bien-fondé de certaines des hypothèses mentionnées plus haut. On peut déjà rejeter celle d'un unique produit fluorescent qui serait obtenu à partir de chacune des catécholamines (16). Nous avons donc entrepris une étude systématique de ces étapes de la réaction, dans le but d'en arriver à une meilleure compréhension de son mécanisme et des facteurs qui peuvent l'affecter.

Mode opératoire et appareils

À moins d'indications contraires, la réaction entre l'adrénaline et l'éthylènediamine fut effectuée selon la technique décrite par Weil-Malherbe et Bone (2).

La fluorescence fut mesurée avec un fluorimètre construit sur place et utilisant le circuit électronique du spectrophotomètre Beckman, modèle B (20). Les filtres employés furent les suivants:

(*a*) Filtre primaire: Corning 3389 et 5113 combinés (5-74) servant à isoler la raie 436 du mercure.

(*b*) Filtre secondaire jaune: Corning 3484 (3-68).

(*c*) Filtre secondaire vert: Corning 4010 (4-64).

Pour les expériences de cinétique, le mélange réactionnel était constitué de 40 ml d'une solution d'adrénaline de 1000 μg/litre et de 40 ml du réactif à l'éthylènediamine décrit plus loin. Des prélèvements de 10 ml furent faits aux temps indiqués, refroidis immédiatement et extraits par 10 ml d'isobutanol pour lecture au fluorimètre.

Pour les mesures de radioactivité, nous avons employé un compteur Geiger (TGC-2) et une échelle Berkeley (Model 2-101 scaler). Les solutions d'adrénaline radioactive ou de ses dérivés furent évaporées dans des cupules en acier inoxydable (Tracerlab E-20).

Réactifs

1. *l*-Adrénaline (Distillation Products). Les solutions furent préparées dans de l'acide acétique 0.2 *N*.

2. Bitartrate de β-C^{14}-*dl*-adrénaline (Atomic Energy of Canada Limited).

3. L'adrénochrome fut fraîchement préparé pour chaque expérience d'après la méthode de Buchnea (oxyde d'argent), telle que décrite par McCarthy (21).

4. Ethylènediamine (Fisher) redistillée.

Les solvants utilisés pour l'extraction furent redistillés avant usage. Tous les autres réactifs étaient chimiquement purs.

Partie expérimentale

pH optimum de la réaction

La réaction entre l'adrénaline ou l'adrénochrome et le chlorhydrate d'éthylènediamine seul ne conduit pas à la formation de matériel fluorescent. Dans le cas de l'adrénaline, si le même mélange est alcalinisé, la réaction s'accomplit normalement, indiquant que l'adrénaline n'a pas subi de transformation appréciable. Si l'on effectue la réaction en milieu alcalin (pH 8.85 à 11.35) et si l'on extrait directement le mélange réactionnel par l'isobutanol, on voit (figure 1A, courbe 2) que le maximum de fluorescence s'obtient dans l'intervalle de pH situé entre 10.2 et 10.4. Il y a évidemment deux facteurs dont puisse dépendre le maximum de fluorescence: (*a*) le pH de la réaction et (*b*) le pH d'extraction.

Nous avons alors préparé une série d'échantillons qui furent soumis à la réaction à pH 10.3 mais qui furent extraits à des pH variant de 3.9 à 11.1. Le meilleur rendement en matériel fluorescent fut obtenu à pH 10.1, comme l'indique la figure 1B.

Dans une autre série d'expériences, la réaction fut effectuée à des pH différents (de 8.7 à 11.45), mais le pH du milieu réactionnel fut amené à 10.1

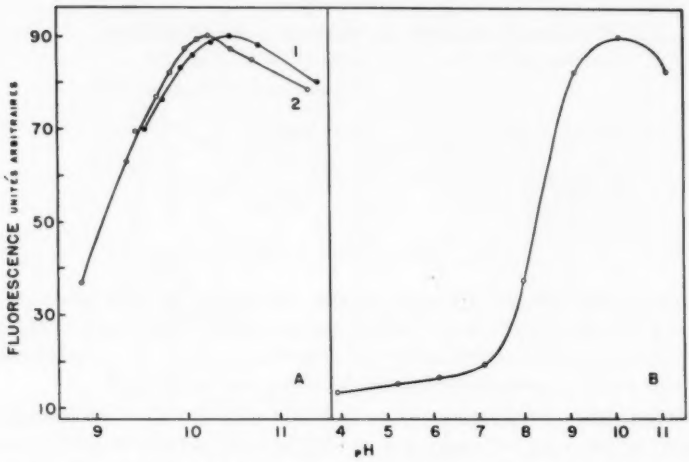


FIG. 1. Influence du pH de réaction et d'extraction sur le rendement en matériel fluorescent extractible.

avant l'extraction. Nous avons trouvé que la réaction à pH 10.5 ± 0.2 donnait le plus fort rendement en matériel fluorescent extractible (figure 1A, courbe 1).

Composition du réactif

Des observations précédentes on peut conclure que la meilleure composition du réactif sera celle permettant d'amener le pH du mélange réactionnel au voisinage de 10.4, tout en assurant une concentration convenable en éthylènediamine. Un mélange de 10 ml d'éthylènediamine (85%) et de 3 ml d'acide chlorhydrique (37%) donne un pH voisin de 10.7. Nous avons donc préparé des dilutions de ce mélange et nous les avons ajoutées à des solutions d'adrénaline. Nous avons trouvé que le rendement maximum en matériel fluorescent s'obtient avec un réactif composé de 10 parties d'éthylènediamine (85%), 3 parties d'acide chlorhydrique (37%) et 42.5 parties d'eau distillée. Une prise de 10 ml de ce réactif ajouté à un volume égal d'une solution d'adrénaline donne un mélange réactionnel contenant 22.9 millimoles d'éthylènediamine partiellement neutralisées pour amener le pH à 10.4 ± 0.1.

Extraction du matériel fluorescent

(*a*) *Choix du solvant.*—Les résultats que nous avons obtenus par chromatographie sur papier (26) ont montré que la quantité de matériel fluorescent extrait augmente avec la polarité des solvants utilisés, i.e. dans l'ordre suivant: éther de pétrole, benzène, éther, acétate d'éthyle, alcools aliphatiques. Parmi ces derniers les alcools *n*- et isobutyliques, *n*- et isoamyliques se sont avérés les plus intéressants. Le tableau I montre que l'isobutanol semble le solvant de choix, tandis que les autres alcools sont équivalents entre eux. De plus,

TABLEAU I

Fluorescence extractible par différents alcools aliphatiques

Solvant, alcool	Fluorescence, unités arbitraires	
	Filtre jaune	Filtre vert
n-Butylique	73	28
Isobutylique	90	33
n-Amylique	77	35
Isoamylique	75	30

la manipulation désagréable des alcools amyliques les fait abandonner au profit des alcools butyliques. À part ces considérations, nous avons préféré l'alcool isobutylique comme agent d'extraction à cause de sa faible solubilité dans l'eau et de sa moindre tendance à former des émulsions.

(*b*) *Efficacité de l'extraction.*—Aux faibles concentrations d'adrénaline, une seule extraction avec relargage par le chlorure de sodium réussit à recouvrer pratiquement tout le matériel fluorescent. Le tableau II fait voir l'intensité

TABLEAU II

Efficacité d'extractions successives

Extractions	Fluorescence, unités arbitraires			
	100 μg/litre		50 μg/litre	
	Sans NaCl	Avec NaCl	Sans NaCl	Avec NaCl
1	58.0	93.0	43.0	66.0
2	21.0	12.5	15.0	5.5
3	8.5	4.5	6.0	0.0
4	3.0	0.0	3.0	0.0
5	1.0	0.0	2.0	0.0
6	0.0	0.0	2.0	0.0

de la fluorescence obtenue par des extractions successives avec des volumes d'alcool isobutylique identiques à celui du mélange à extraire. Il faut cependant noter que ces valeurs ne représentent pas nécessairement le contenu réel en adrénaline. La figure 2 montre la proportion de substances radioactives recouvrées par des extractions successives, comme pour les mesures de fluorescence précédentes. Au moins 20% de l'adrénaline originale reste dans la phase aqueuse sous forme d'adrénaline non transformée ou de dérivés non extractibles même après saturation de la phase aqueuse par le chlorure de sodium. On trouvera des détails supplémentaires à ce sujet dans une communication ultérieure (26).

Durée et température de la réaction

La figure 3 fait voir l'influence de la température sur la réaction entre l'adrénaline et le réactif à l'éthylènediamine. Quand la réaction se fait à 50° C, le maximum de fluorescence extractible est généralement atteint entre

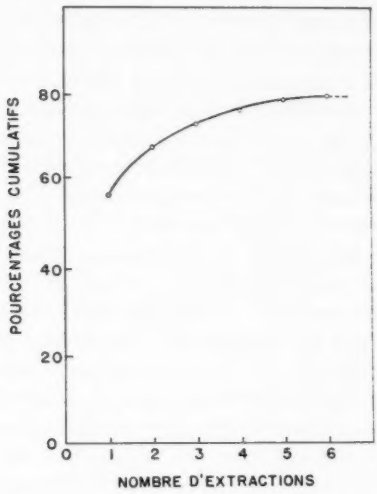


FIG. 2. Pourcentages cumulatifs de matériel radioactif (et fluorescent) obtenu par des extractions successives.

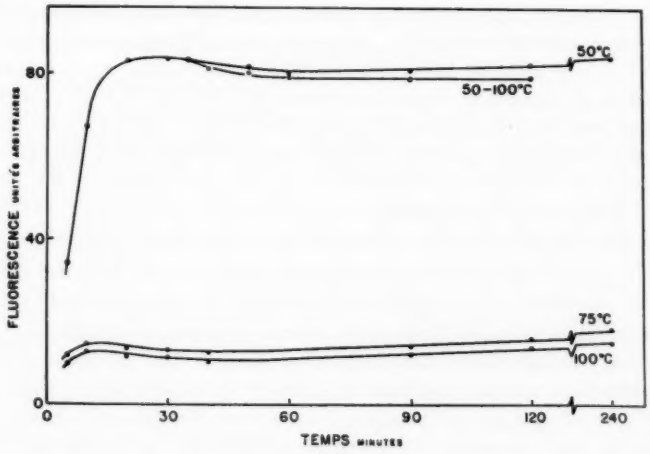


FIG. 3. Influence de la température et du temps sur le rendement en fluorescence totale extractible.

20 et 30 minutes. L'addition d'un excès d'air ou la substitution d'adrénochrome n'améliorent pas les résultats. Notons cependant, comme nous le mentionnerons plus loin, que le barbotage d'oxygène dans le mélange réactionnel situe le plateau de fluorescence maximum à un niveau plus élevé.

À des températures supérieures (75° C et 100° C), le maximum de fluorescence extractible est considérablement abaissé, bien qu'il soit atteint plus rapidement. Ce comportement peut s'expliquer par au moins deux hypothèses: (*a*) à ces températures, le milieu réactionnel ne contiendrait plus d'oxygène dissous, inhibant ainsi la première étape de la réaction (oxydation) ou (*b*) le matériel fluorescent produit au cours de la réaction serait thermolabile.

La réaction a donc été reprise (*a*) en présence d'un excès d'air et (*b*) avec de l'adrénochrome fraîchement préparé. En aucun cas, il n'y eut augmentation notable de la fluorescence. Dans une autre expérience, la réaction fut effectuée à 50° C durant 35 minutes, puis le mélange réactionel fut porté à 100° C pendant 85 minutes. La fluorescence extractible demeura pratiquement constante durant ce laps de temps (figure 3, courbe 50°–100° C). La diminution de fluorescence observée au cours de la réaction faite à 100° C (ou à 75° C) ne peut donc pas s'expliquer par l'instabilité des produits de la réaction. On pourrait aussi suggérer qu'à ces températures relativement élevées, il y ait formation de produits différents de ceux qui constituent en fait le matériel fluorescent, comme nous l'avons démontré précédemment (16). Cette hypothèse a été vérifiée en séparant par chromatographie sur papier les produits obtenus par réaction à 50° et à 100° C respectivement. La zone correspondant au produit principal fut éluée à l'eau et la solution extraite par l'isobutanol. La fluorescence de ces extraits fut comparée à celle d'extraits contenant tous les produits extractibles, pour des réactions effectuées aux deux températures mentionnées. Le rapport de la fluorescence totale extractible à 50° C sur celle de la réaction à 100° C est voisin de 3, tandis que le rapport de la fluorescence du produit principal est d'environ 2. Cette observation démontre un changement dans la proportion relative des constituants du matériel fluorescent quand la réaction est portée de 50° à 100° C.

Oxydation préliminaire en quinone

Si l'on effectue la réaction avec une quantité assez grande d'adrénaline (v.g. 5 g/litre), en solution saturée d'air, le rendement en matériel fluorescent est faible. En outre, on récupère environ 35% de l'adrénaline de départ.

Quand on ajoute à de l'éthylènediamine chauffée à reflux une solution d'adrénaline préalablement dégazée, on n'observe pas de fluorescence tant que l'oxygène est exclus, bien que la solution se colore légèrement en bleu. La figure 4 illustre l'augmentation de fluorescence obtenue en faisant barboter de l'oxygène dans le milieu réactionnel (courbe 1) comparativement à la technique habituelle (courbe 2). On y voit aussi que l'oxygène a peu d'influence sur la réaction une fois le maximum atteint. D'autre part, si l'on ajoute l'oxygène avant d'atteindre le maximum de fluorescence, on obtient un palier intermédiaire entre celui de la réaction ordinaire et celui d'une

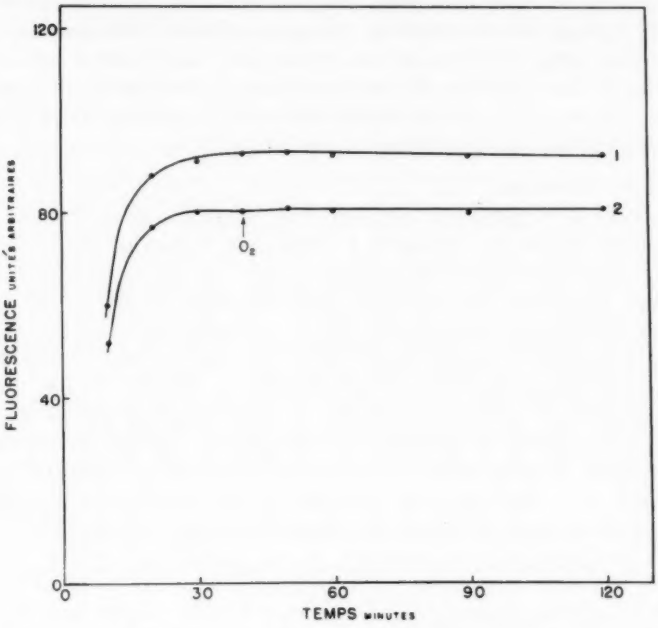


FIG. 4. Influence de l'oxygène sur le rendement en fluorescence totale extractible.

réaction effectuée en présence d'oxygène dès le début. Quand on remplace l'adrénaline par une solution équivalente d'adrénochrome fraîchement préparé, la réaction reste essentiellement la même.

Influence de différents ions

Nous avons examiné l'influence des sels suivants sur la réaction entre l'adrénaline et l'éthylènediamine: nitrate d'argent, chlorure de zinc, chlorure ferrique, sulfate cuivrique, sulfate d'aluminium et molybdate d'ammonium. Le rapport moléculaire sel/adrénaline était de 2 à 1. L'effet des trois derniers sels est illustré dans le tableau III. Le rôle des ions Cu^{++} et Al^{+++}

TABLEAU III

Influence combinée de la lumière, de l'oxygène et de différents ions sur le rendement en matériel fluorescent extractible

Réaction	Sans addition d'oxygène			Avec oxygène
	Obscurité	Lumière diffuse	Lumière ultraviolette	Lumière diffuse
Ordinaire	70	63	54	89.2
Avec $CuSO_4$		66		85
Avec $Al_2(SO_4)_3$		64.5		85
Avec $(NH_4)_2MoO_4$		89.5		83.5

étant facilement explicable (voir Discussion), c'est l'ion molybdate qui a attiré d'avantage notre attention. L'augmentation de fluorescence obtenue par addition d'une solution de molybdate est comparable à celle que l'on obtient en faisant barboter de l'oxygène dans le mélange dès le début de la réaction. Il est à remarquer cependant que la combinaison de ces deux facteurs n'améliore pas le rendement (tableau III).

Influence de la lumière

L'influence de la lumière a été étudiée en effectuant la réaction en lumière ultraviolette, en lumière diffuse et à l'obscurité, toutes les autres conditions expérimentales demeurant identiques à celle de la technique habituelle. Le tableau III montre que la fluorescence croît dans l'ordre précité. Cependant, comme l'indique la figure 5 (courbe 1) la lumière ultraviolette (exposition directe à une lampe à mercure G.E. AH-4, 100 watts) a peu d'influence sur le maximum de fluorescence extractible obtenue en lumière diffuse. Au cours de cette expérience, la réaction, effectuée en lumière diffuse durant 40 minutes, fut soumise à l'effet de la lumière ultraviolette pour une période additionnelle de 80 minutes. Notons qu'au contraire, l'éclairage par la lumière ultraviolette dès le début de la réaction a eu pour effet de diminuer de façon appréciable le rendement en matériel fluorescent extractible (figure 5, courbe 2).

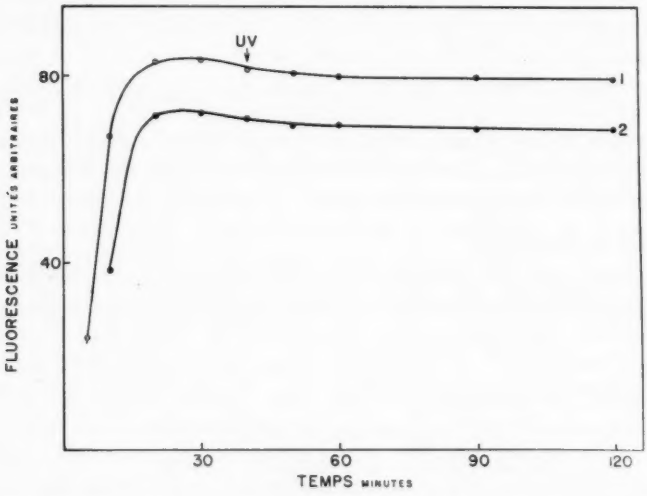


FIG. 5. Influence de la lumière ultraviolette sur le rendement en fluorescence totale extractible.

Discussion

Les résultats accumulés au cours du présent travail ne visaient pas nécessairement à l'élaboration d'une technique analytique utilisable aux basses teneurs en catécholamines rencontrées dans les liquides biologiques. Cependant, beaucoup d'entre eux ont pu servir d'indices précieux à cette fin (22).

Nos travaux confirment le besoin d'oxygène ou d'un autre agent oxydant dans le mélange réactionnel, du moins au début de la réaction, puisqu'il apparait sans effet une fois le maximum atteint. L'oxydation étant une étape essentielle, il faut donc en uniformiser soigneusement les conditions de façon à ce que les résultats soient reproductibles.

Selon nos observations, la réaction ne procède normalement qu'en milieu alcalin. Elle s'effectue avec un rendement acceptable si l'on emploie l'éthylènediamine seule (pH 12.3), tandis qu'elle est pratiquement nulle avec le chlorhydrate d'éthylènediamine seul (pH 4.3). Le rendement de la réaction en matériel fluorescent extractible est à son maximum à pH 10.5, tandis que l'extraction se fait le plus complètement à pH 10.1 ± 0.1. Comme ces valeurs de pH sont assez rapprochées et que l'extraction se fait encore avec un rendement appréciable à pH 10.5, il ne semble donc pas nécessaire d'ajuster l'alcalinité avant l'extraction. Nous avons opté pour un réactif qui donne un pH intermédiaire de 10.3 ± 0.1. Les teneurs en acide chlorhydrique et en éthylènediamine nécessaires pour obtenir ces valeurs de pH sont d'ailleurs assez voisines de la composition du réactif suggéré par Weil-Malherbe et Bone (2).

L'étude de l'effet de la température sur le rendement total en matériel fluorescent extractible montre que le maximum s'obtient plus rapidement à mesure que l'on opère à des températures plus élevées, mais que l'intensité de ce maximum diminue avec l'accroissement de la température. Il y a donc avantage à effectuer la réaction à 50° C plutôt qu'à 100° C et même à la température de la pièce, bien que le terme de la réaction soit retardé. Nous avons d'ailleurs noté en certaines occasions qu'une période de 20 minutes à 50° C n'était pas suffisante. C'est pourquoi, dans les études de cinétique, nous avons généralement laissé la réaction se poursuivre durant 30 ou 40 minutes avant de changer les conditions expérimentales. Comme la fluorescence obtenue aux différentes températures ne décroît que très lentement une fois le maximum atteint, il semble donc que le matériel fluorescent une fois formé soit assez stable (pour au moins 2 heures).

Erne et Canbäck (13) ont démontré l'effet destructeur de la lumière ultraviolette sur le matériel fluorescent obtenu à partir de la noradrénaline ayant réagi avec l'éthylènediamine. Nous avons examiné l'effet de la lumière sur la réaction entre l'adrénaline et la même diamine. La fluorescence décroît d'après les conditions d'éclairage suivantes: obscurité, lumière diffuse, lumière ultraviolette. Il est à noter que la fluorescence ne diminue que très peu par exposition à la lumière ultraviolette une fois le maximum atteint. Il semble que contrairement à la noradrénaline, la fluorescence du dérivé de l'adrénaline soit relativement stable, ce qui pourrait être mis à profit dans un dosage différentiel de ces deux catécholamines. À cause de la difficulté d'uniformiser les conditions d'éclairage et de l'effet nocif de la lumière sur la noradrénaline, le mode opératoire indiqué est de laisser la réaction s'effectuer à l'obscurité, si l'on veut l'appliquer au dosage différentiel des deux catécholamines.

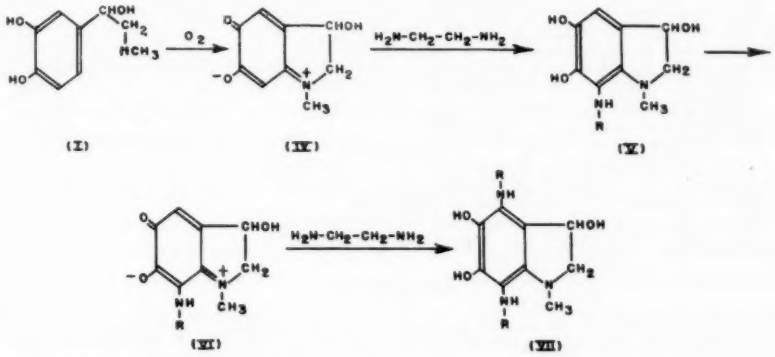
Bien que les produits de la réaction soient relativement stables vis-à-vis de l'oxygène, de la température ou de la lumière ultraviolette, une fois le maximum de fluorescence atteint, nous avons noté un abaissement parfois considérable du maximum de la fluorescence par élévation de la température ou par exposition à la lumière ultraviolette *dès le début de la réaction*. La diminution semble attribuable à la destruction de certains composés intermédiaires suivie de la formation de produits non fluorescents. Cette déviation dans la suite des réactions se ferait au détriment du principal produit fluorescent jaune obtenu dans les conditions ordinaires.

Aux concentrations étudiées, des mesures de fluorescence accompagnées de détermination de radioactivité font voir que, même par des extractions répétées, avec ou sans relargage, on ne peut extraire plus de 80% des dérivés de l'adrénaline. On peut donc conclure que la réaction donne lieu à la formation d'autres dérivés, fluorescents ou non, qui ne sont pas extractibles en milieu alcalin. Cet aspect a d'ailleurs été étudié par nous au cours de nos expériences de chromatographie sur papier et fait le sujet d'une communication subséquente (26).

L'augmentation de la fluorescence causée par l'addition de divers ions, déjà signalée par nous (18) et étudiée dans le présent travail, pourrait s'expliquer soit par la formation de complexes auto-oxydables, soit par un effet purement catalytique. Chaix et ses collaborateurs (23, 24), qui ont étudié l'oxydation de l'adrénaline en présence d'ions métalliques, ont signalé la formation d'un complexe adrénaline–cuivre auto-oxydable. Ils ont de plus démontré que l'adrénochrome est auto-oxydable, mais que la réaction n'est pas affectée par la présence du cuivre (25). Il est à remarquer cependant que, dans la présente réaction, les ions (sauf MoO_4^{--}) sont complexés davantage par l'éthylènediamine que par les catécholamines et qu'ils sont ainsi peut-être plus actifs comme catalyseurs d'oxydation. Les travaux de Brackman et Havinga (27) ont en effet mis en évidence le pouvoir oxydant des complexes amines-cuivre solubles. Ceux-ci peuvent transformer les phénols en catéchols et en ortho-quinones correspondantes, les quinones réagissant alors avec les amines pour former des produits d'addition suivant le mode habituel de réaction des quinones. Quant au molybdate d'ammonium, en raison de son influence comparable aux ions précédemment cités, on pourrait lui attribuer un rôle analogue. Cohen (28) a déjà d'ailleurs isolé un complexe adrénalino-molybdique à caractère phénolique. Il ne faut cependant pas ignorer la possibilité d'une autre voie d'oxydation, si l'on se rappelle l'observation de Brackman et Havinga (27) que, dans l'oxydation des catéchols avec l'ion Cu^{++}, une amine et le molybdate d'ammonium, on obtient les para-quinone-imines plus facilement que les ortho-quinone-imines isomères.

Comme nous l'avons vu, l'oxydation de l'adrénaline en adrénochrome est une étape nécessaire dans la présente réaction. Or nous avons démontré par chromatographie sur papier (16, 26) que les produits de la réaction sont

qualitativement les mêmes que l'on parte soit de l'adrénaline, soit de l'adrénochrome. Nous pouvons donc en conclure que la réaction s'effectue en réalité entre l'adrénochrome et la diamine. D'autre part, les travaux de Harley-Mason (30) et de Rozum (31) ont montré que l'adrénochrome est une para-quinone-imine et non une ortho-quinone. En effet, l'adrénochrome ne forme pas de phénazine avec l'ortho-phénylènediamine et ne donne qu'une monoxime et qu'une semi-carbazone. De plus, les quinones subissent généralement plus facilement l'addition 1,4 que la condensation avec une diamine pour former des quinoxalines. Cette dernière réaction requiert d'ailleurs des conditions beaucoup plus énergiques, en particulier avec l'éthylènediamine (29). Devant ces faits, la réaction, ainsi que la structure hypothétique du produit principal, telles que proposées par Weil-Malherbe et Bone (2) ne nous semblent pas les plus probables, ce qui nous amène à soumettre les réactions suivantes comme autres voies possibles:



R = —CH_2—CH_2—NH_2

L'adrénaline (I) serait d'abord oxydée en adrénochrome (IV) qui subirait l'addition 1,4 d'une première molécule d'éthylènediamine pour donner un amino-catéchol N-substitué (V). Ce nouveau composé serait oxydé par l'oxygène ou par l'adrénochrome pour former la quinone (VI) pouvant additionner une deuxième molécule d'éthylènediamine avec formation du diamino-catéchol (VII). Ce dernier pourrait de nouveau donner une quinone avec possibilité de combinaison avec une troisième molécule d'éthylènediamine, de cyclisation en tétrahydroquinoxaline ou encore d'une réaction à la manière des amino-phénols en formant des dérivés para-oxaziniques. Ces dernières possibilités font présentement l'objet d'une autre investigation.

Remerciements

Les auteurs expriment leur gratitude au Conseil National des Recherches pour l'octroi de bourses d'études à l'un d'eux (L.-P. J.). Ils remercient également le Docteur G. Sobolewski pour ses judicieux conseils et pour l'intérêt constant qu'il a porté à ce travail.

Bibliographie

1. NATELSON, S., LUGOVOY, J. K. et PINCUS, J. B. Arch. Biochem. **23**, 157 (1949).
2. WEIL-MALHERBE, H. et BONE, A. D. Biochem. J. **51**, 311 (1952).
3. WEIL-MALHERBE, H. et BONE, A. D. Lancet, **264**, 974 (1953).
4. MANGER, W. M., BALDES, E. J., FLOCK, E. V., BOLLMAN, J. L., BERKSON, J. et JACOBS, M. Proc. Staff Meetings Mayo Clinic, **28**, 526 (1953).
5. RICHARDSON, J. A., RICHARDSON, A. K. et BRODIE, O. J. J. Lab. Clin. Med. **47**, 832 (1956).
6. ARONOW, L. et HOWARD, F. A. Federation Proc. **14**, 315 (1955).
7. KÄGI, J., BURGER, M. et GIGER, K. Arch. exptl. Pathol. Pharmakol. **230**, 470 (1957).
8. VALK, A. de T. et PRICE, H. L. J. Clin. Invest. **35**, 837 (1956).
9. SOURKES, T. L. et DRUJAN, B. D. Can. J. Biochem. and Physiol. **35**, 711 (1957).
10. ABELIN, I. et GOLDSTEIN, M. Biochem. Z. **327**, 72 (1955).
11. SOBEL, C. et HENRY, R. J. Am. J. Clin. Pathol. **27**, 240 (1957).
12. BURN, G. P. et FIELD, E. O. Nature, **178**, 542 (1956).
13. ERNE, K. et CANBÄCK, T. J. Pharm. and Pharmacol. **7**, 248 (1955).
14. ELLMAN, G. L. Nature, **181**, 768 (1958).
15. GRAY, I. et YOUNG, J. G. Clin. Chem. **3**, 239 (1957).
16. NADEAU, G. et JOLY, L. P. Nature, **182**, 180 (1958).
17. BONE, A. D. J. Med. Lab. Tech. **11**, 212 (1952).
18. NADEAU, G., JOLY, L. P. et SOBOLEWSKI, G. Nature, **181**, 1061 (1958).
19. MILLAR, R. A. J. Pharmacol. Exptl. Therap. **118**, 435 (1956).
20. JOLY, L. P., SOBOLEWSKI, G. et NADEAU, G. Clin. Chem. **4**, 286 (1958).
21. MCCARTHY, C. L. Chim. et ind. **55**, 435 (1946).
22. NADEAU, G. et SOBOLEWSKI, G. À publier.
23. CHAIX, P., CHAUVET, J. et JEZEQUEL, J. Biochem. et Biophys. Acta, **4**, 471 (1950).
24. CHAIX, P., MORIN, G. A. et JEZEQUEL, J. Biochem. et Biophys. Acta, **5**, 472 (1950).
25. GAUTHERON, D. et CHAIX, P. Biochem. et Biophys. Acta, **13**, 299 (1954).
26. NADEAU, G. et JOLY, L. P. À publier.
27. BRACKMAN, W. et HAVINGA, E. Rec. trav. chim. **74**, 937, 1021, 1070, 1107 (1955).
28. COHEN, G. N. Bull. soc. chim. biol. **XXVII**, 237 (1945).
29. GRIGNARD, V. Traité de chimie organique. Tome XXI. Masson et Cie, Paris. 1953.
30. HARLEY-MASON, J. Experientia, **4**, 307 (1948).
31. ROZUM, Y. S. Biokhimiya, **17**, 476 (1952).
32. GOLDFIEN, A. et KARLER, R. Science, **127**, 1292 (1958).

Addendum

Depuis la rédaction de ce travail, la communication récente de Goldfien et Karler (32) nous a été signalée. La stabilité de la fluorescence de l'adrénaline et l'instabilité de celle de la noradrénaline vis-à-vis de la lumière ultraviolette y sont confirmées.

THE ACTIVATION AND DETERMINATION OF PLASMIN IN THE RAT[1]

S. W. LEVY[2]

Abstract

The presence of a proteolytic enzyme precursor (plasminogen) in the euglobulin fraction of rat plasma was demonstrated and optimum conditions for its activation and determination were studied. Rat plasmin activity was estimated (casein-digestion method) following activation of the enzyme with streptokinase-activated human serum euglobulin under various conditions of temperature, time, activator concentration, and pH. The activation and subsequent inactivation of plasmin in the presence of activated human euglobulin were shown to be accompanied by the appearance of acid-soluble, ultraviolet-absorbing substances in the activation mixture. The results of these studies were discussed in terms of the concept that the activation and inactivation of rat plasmin are essentially autocatalytic reactions and represent stages in the progressive degradation of the plasminogen molecule.

Introduction

The occurrence of one or more proteolytic enzymes in the blood of animals and humans has been widely investigated (1, 2). Changes in blood proteolytic activity have been observed in a variety of pathological and experimental states (3–10) and recent investigations on the use of proteolytic enzymes or activators in the treatment of thromboembolic and inflammatory diseases (11) have aroused considerable interest. The protease commonly studied exists in human plasma in an inactive form (profibrinolysin, plasminogen) which may be converted to the active enzyme (fibrinolysin, plasmin) under the influence of chloroform and other lipid solvents (12–15), glycerol (16), trypsin (15, 16, 17), bacterial kinases (18–21), and extracts of tissues (22–26), and urine (27, 28, 29). Spontaneous fibrinolytic and proteolytic activities have also been observed in enzyme preparations from various species and following storage at 5° C (18, 25, 30, 31). Many difficulties have been encountered in studies on plasmin activity owing to the simultaneous presence in plasma of inhibitors (32–36) and activators (20, 37, 38, 39) of the enzyme, its strong adsorption to fibrin (1, 40, 41), and the low levels or complete absence of streptokinase-activated plasmin activity in experimental animals (18, 30, 42). These difficulties have been overcome in part by the use of crude or purified plasma extracts containing the enzyme precursor (41, 43) and the use of plasminogen-free or synthetic substrates (16, 38, 44, 45).

The observation in recent years that human plasma contains relatively high concentrations of a proactivator, which is converted by streptokinase to a potent activator of plasminogen (2, 20, 39), has served both to elucidate the mechanism of enzyme activation and to permit studies to be carried out in

[1]Manuscript received September 29, 1958.
Contribution from the Departments of Rheumatology and Biochemistry, Hôtel-Dieu Hospital, Montreal, Que. Supported by a grant-in-aid from the Ministry of Health, Province of Quebec and the Department of National Health and Welfare, Ottawa, Canada.
[2]Departments of Rheumatology and Biochemistry, Hôtel-Dieu Hospital, Montreal, Que.

species which exhibit little or no spontaneous or streptokinase-activated plasmin activity. Initial attempts in our laboratory to estimate plasmin activity in the rat by means of activation with streptokinase and staphylokinase yielded low activities with staphylokinase and none with streptokinase. The introduction of "proactivator" in the form of a euglobulin extract from human serum was then shown to provide the basis for a suitable method for estimating plasmin activity in small volumes of rat plasma.

Materials and Methods

Proactivator.—Pooled human serum was diluted to 20 volumes with 0.016% acetic acid (30), centrifuged, and the euglobulin precipitate taken up in 1/5 serum volume of 0.1 *M* phosphate–saline buffer (pH 7.6). This proactivator stock solution was divided into several small portions (3 ml) and stored at −20° C.

Streptokinase (*Varidase*)*—was dissolved in distilled water (10 mg/ml) and stored at −20° C.

Activator.—Proactivator stock solution (4 volumes) was incubated with streptokinase solution (1 volume) at 37° C for 10 minutes, chilled or frozen, and used as soon as possible to activate the plasminogen. This ratio of streptokinase:proactivator was found by experiment to give the highest yield of activator under the conditions employed. Higher concentrations of streptokinase had no effect on the level of activator produced. In agreement with Mullertz (46) activator solutions were found to be relatively unstable compared with proactivator, plasminogen, or plasmin.

Plasminogen (*rat*).—Blood was drawn from the jugular vein of ether-anaesthetized rats into 1/10 volume of sodium citrate solution (3.8%), chilled, centrifuged, and the plasma removed. The euglobulin fraction was precipitated with 19 volumes of dilute acetic acid (cf. "Proactivator") and made up in citrated phosphate–saline buffer (pH 8.0) to the original plasma volume.

Plasmin (*rat*).—The activation of plasminogen was accomplished by incubating it with activator solution under various conditions of temperature, time, pH, and activator concentration. Optimum conditions for the activation of plasminogen and the activity of plasmin were determined in the experiments to be described. An active preparation of plasmin was found to be stable for several months at −20° C.

Casein.—Soluble, purified casein (Fisher) was used as the substrate for the assay of plasmin. The casein was dissolved slowly in hot, citrated phosphate–saline buffer (pH 8.0) to a concentration of 3.0% and the solution incubated in a boiling-water bath for 15 minutes in order to destroy the activity of milk proteases and to dissolve the casein completely. A large volume of casein was prepared and stored in separate flasks at −20° C for each series of experiments in order to ensure a constant substrate composition.

*A generous supply of Varidase of a single lot number was donated by the Lederle Laboratories Division of the North American Cyanamid Company.

Buffers.— For routine work in dissolving euglobulin precipitates and the casein substrate and as a diluting medium, the buffers used were NaH_2PO_4–Na_2HPO_4 (0.1 *M*), pH 7.6 and 8.0 (47), dissolved in 0.9% NaCl solution. Sodium citrate was added to a concentration of 3.8% in order to prevent the formation of fibrin clots in rat-plasma euglobulin solutions. The pH was adjusted and sodium merthiolate (1:1000) (1.0 ml/liter) was added as a preservative. The buffers used in experiments on the effects of pH on the activity of plasmin were: citrate–phosphate (pH 5.4), phosphate (pH 6.3 to 7.9), boric acid–borate (pH 8.2–8.7), and borate–NaOH (pH 9.4) (47). All buffers were made up in 0.9% NaCl solution.

Perchloric acid.—As recommended by Mullertz (39), $HClO_4$ (1.7 *M*) was found to be superior to trichloroacetic acid as a deproteinizing agent. Routinely, 2.0 ml of the reaction mixture were added to 2.0 ml of 1.7 *M* $HClO_4$. The tubes were shaken, allowed to stand at least 1 hour, centrifuged at 3000 r.p.m. for 15 minutes, and the supernatants decanted for assay.

The assay of plasmin.—Activated plasminogen solutions were diluted with cold, citrated phosphate–saline buffer, pH 8.0, (usually 0.2 ml of plasmin was added to 3.0 ml of buffer) and brought to temperature equilibrium with casein for 5 minutes at 37° C. An equal volume of casein was added and the solutions were mixed and incubated at 37° C. Samples (2.0 ml) of the digestion mixtures were removed into 1.7 *M* $HClO_4$ (2.0 ml) at zero hour and, thereafter, at two regular intervals of 1 or 1½ hours' duration. The deproteinized samples were read in the Beckman Model DU spectrophotometer for substances absorbing in the ultraviolet at a wave length of 275 mμ. Standard Beckman cuvettes, having a 1.0-cm light path, were used. Results were expressed as the increase in optical density per hour of casein digestion.

Results

Following the development of suitable conditions for the activation of rat plasminogen and the digestion of casein, it was shown that the increase in optical density of deproteinized samples removed from the digestion mixture was directly proportional to the digestion time for a period of at least 3 hours (Fig. 1*a*). A solution of rat plasminogen (1 ml/ml plasma, 0.4 ml) was incubated with activator solution (0.004 ml) at 37° C for 3 hours. Phosphate–saline buffer (pH 8.0, 6.0 ml) was added and, after temperature equilibration, 6.4 ml of casein solution (3.0%) was added and mixed. The deproteinized samples removed from the digestion tube were read at 275 mμ against a perchloric acid blank.

Portions of the same solution of plasmin used in the above experiment (obtained originally from the pooled, citrated blood of three rats) were diluted to a series of lower concentrations with phosphate–saline buffer (pH 8.0) and the proportionality between enzyme concentration and the rate of increase in optical density was demonstrated (Fig. 1*b*). The digestion curves for all concentrations of the enzyme exhibited linearity during the 3-hour period of digestion.

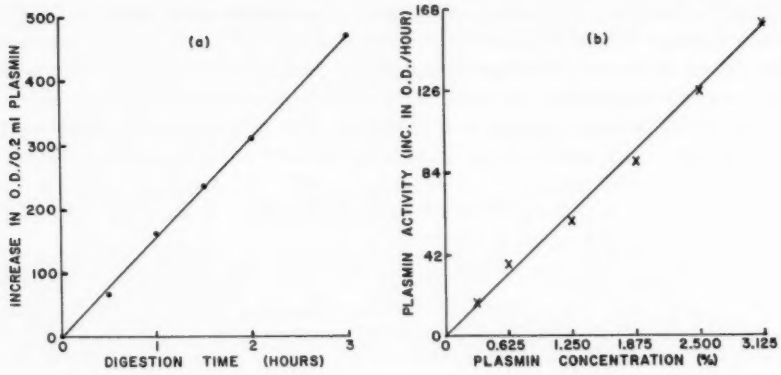


FIG. 1. The activity of rat plasmin. (*a*) The relation between increase in optical density of casein digests (deproteinized) and digestion time ($\lambda = 275$ mμ); (*b*) the relation between enzyme concentration and hourly rate of digestion of casein.

Experiments in which a higher concentration of activator (3.0%, v/v) and a shorter activation time (1½ hours) were used showed the same linear relationships between the increase in extinction on the one hand and digestion time or enzyme concentration on the other (Fig. 2). Control tubes containing equivalent amounts of activator but no enzyme exhibited little or no casein-splitting activity. Since these conditions yielded considerably higher activities than those described above, they were ultimately adopted for the routine determination of rat plasmin activity.

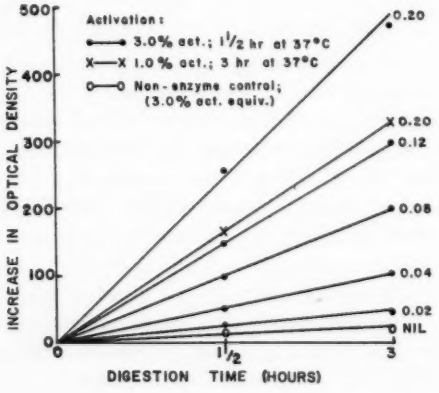


FIG. 2. The increase in optical density with time of casein-digestion mixtures at various concentrations of rat plasmin and following two procedures for activation of the plasminogen.

The Optimum pH of Rat Plasmin

The euglobulin precipitate (plasminogen) from rat plasma was dissolved in citrated phosphate–saline buffer (1 ml/ml plasma, pH 7.4) and activated with streptokinase-activated human euglobulin (1.0%, v/v; 3 hours at 37° C). Aliquots (0.2 ml) were removed into cold buffer solutions (3.0 ml) of different pH values with a range of 5.4 to 9.4. Casein solutions (3.0%) previously prepared in the same buffers were brought separately to temperature equilibrium at 37° C and 3.2 ml were added to the enzyme solutions of the same pH. Samples were removed into 1.7 *M* $HClO_4$ at 0, 1½, and 3 hours following addition of the casein. Proportionality between the increase in optical density of deproteinized samples and the digestion time was observed in all samples except those at pH 8.7 and 9.4 where a slight falling-off in the digestion rate occurred during the second 1½-hour period.

The effect of pH on the activity of plasmin is shown in Fig. 3. Maximum activity of the enzyme occurred between pH 7.8 and 8.2 with a somewhat more rapid decrease below than above this range. Similar results have been obtained with bovine and human plasmin by other investigators (cf. 39).

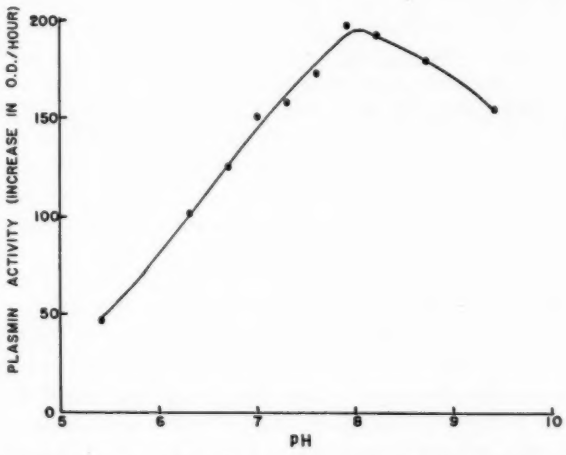


FIG. 3. Effect of pH on the activity of rat plasmin. Points on the curve represent rates of digestion of casein at the pH values shown.

Effects of Temperature and Incubation Time on the Activation of Rat Plasminogen

The conditions described by Mullertz (39) for the activation of ox plasminogen (extracted from fibrin) were found to be inadequate for the activation of rat-plasma euglobulin solutions. Whereas ox plasminogen was maximally activated by the human activator (1.0%, v/v) following incubation for 2 hours at 22° C, rat plasminogen solutions required more prolonged incubation at 37° C with the same concentration of activator. Figure 4 describes the

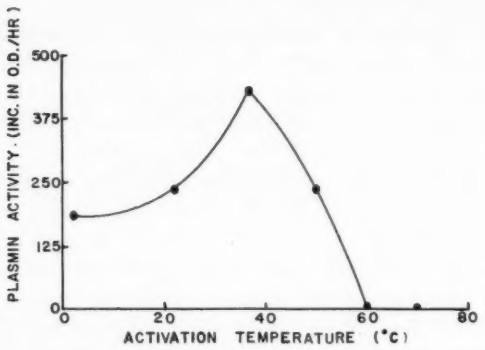


FIG. 4. Effect of temperature on the activity of generated rat plasmin after preliminary activation for 2 hours at 22° C. Incubation time, 30 minutes; activator concentration, 1.0% (v/v).

results of an experiment in which rat plasminogen solutions were preincubated with activator (1.0%, v/v) for a period of 2 hours at 22° C, chilled immediately, and subsequently incubated for 30 minutes at the temperatures shown. The estimation of plasmin activity in these samples revealed that the activation of plasminogen was incomplete following the initial incubation period of 2 hours at 22° C. After the additional incubation period of 30 minutes the activity of the samples was increased over the 2° C control by about 20% at 22° C and by more than 125% at 37° C. At 50° C, the plasmin activity was again only 20% higher than the control and at 60° C and 70° C complete inactivation of the enzyme had occurred. Control tubes containing activator solutions alone diluted to the same extent as those present in the rat enzyme solutions and incubated at these temperatures exhibited no proteolytic activity.

These results showed that the rate of activation of rat plasminogen was highest at 37° C relative to the actual destruction of the enzyme, but gave no indication as to the course of activation with time or the time required for maximum activation of the enzyme to occur. An experiment was therefore performed in which samples of plasminogen were incubated with activator (1.0%, v/v) at 2° C, 25° C, and 37° C, and aliquots removed at various times and frozen immediately in acetone at −20° C. An aliquot removed from the 2° C activation mixture immediately following addition of the activator was taken as the control. The samples of plasmin were collected and stored at −20° C until the casein digestions and assays could be performed. At the end of 6 hours, additional amounts of activator (0.5%, final concentration) were added to the activation tubes maintained at 25° C and 37° C and aliquots removed and frozen 2 and 8 hours later in order to reveal the possible deterioration of the proenzyme or activator.

The casein-digestion curves for this experiment are shown in Fig. 5*a* and it can be seen that plasminogen solutions activated at 2° C and 25° C yielded concave digestion curves whereas those activated at 37° C gave linearity of

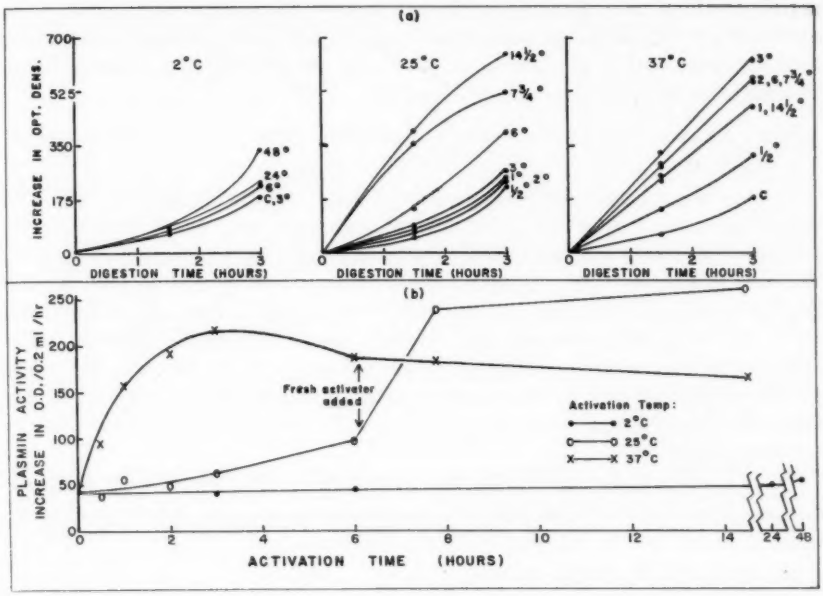


FIG. 5. Effects of temperature and incubation time on the activation of rat plasmin. (*a*) Increase in optical density of casein-digestion mixtures with time. (*b*) Each point on the curves represents the hourly rate of digestion of casein by an aliquot of an activation mixture maintained at the temperature and for the time shown. Activator concentration, 1.0% (v/v).

digestion with time. These results suggest that activation of the enzyme continued to occur at a high rate during the course of digestion in the case of plasminogen samples activated at the lower temperatures. The linearity of digestion observed with plasminogen samples activated at 37° C, however, cannot readily be interpreted to mean either that activation of the available proenzyme was complete or that all of the activator was destroyed or used up in the activation process, since the digestion rate (plasmin activity) varied directly as the time of activation for a period of 3 hours. This is seen more clearly in Fig. 5*b* where the digestion rates are plotted as a function of the plasminogen activation times (see Discussion).

The curves shown in Fig. 5*b* reveal that: (*a*) At 2° C the activation of plasminogen was negligible for as long as 24 hours. (*b*) At 25° C activation progressed slowly and linearly for at least 6 hours. The addition of fresh activator (0.5%, v/v) at this time resulted in a rapid increase in the rate and extent of activation during the next 2 hours indicating that the plasminogen had not been exhausted or destroyed during the preceding 6 hours. The highest activity attained was somewhat higher than that of the sample activated at 37° C. (*c*) The rate of activation was highest at 37° C, the peak of plasmin

activity having been attained in $2\frac{1}{2}$ to 3 hours. At the end of 6 hours, however, the activity declined somewhat owing to the exhaustion of plasminogen and the inactivation of plasmin as shown by the evident lack of response to the addition of fresh activator (0.5%, v/v).

Effects of Activator Concentration and Incubation Time (37° C) on Plasminogen Activation

To explore further the mechanism of plasminogen activation and the optimum conditions for activation of the proenzyme, several experiments were performed in which activation was studied as a function of activator concentration and incubation time. Plasminogen solutions were incubated with various concentrations of activator (0.5 to 5.0%, v/v) at 37° C and pH 8.0, and aliquots (0.2 ml) were removed into ice-cold, citrated phosphate–saline buffer (pH 8.0, 3.0 ml) at various times (0 to 6 hours) after the addition of activator. In the experiment to be discussed a series of control tubes containing the following constituents were treated and sampled in the same manner as the activation mixtures:

(*a*) Non-plasminogen controls: three tubes containing amounts of activator equivalent to 1.0, 3.0, and 5.0% (v/v) of the plasminogen in the activation tubes;

(*b*) plasminogen alone;

(*c*) plasminogen + streptokinase (amount equivalent to that contained in 3.0% activator);

(*d*) plasminogen + proactivator (amount equivalent to that contained in 3.0% activator);

(*e*) plasminogen + boiled activator (3.0%, v/v);

(*f*) boiled plasminogen + activator (3.0%, v/v).

None of the samples withdrawn from these tubes over a period of 3 hours of incubation exhibited any proteolytic activity toward casein.

The results of a representative experiment are shown in Fig. 6, where the plasmin activity developed is plotted as a function of the activation time at each of the six concentrations of activator employed. The activities of aliquots removed from the activation tubes immediately following the addition of plasminogen to the activator were taken as zero-hour controls and all subsequent values were adjusted in terms of these activities taken as zero at each concentration of activator. It should be mentioned that these control activities varied from 2 to 100 optical density units per hour directly as the activator concentration and, therefore, represented a considerable proportion of the activities observed at high activator concentrations. Since the non-plasminogen controls containing activator exhibited no digestive activity toward casein, it was felt that the activities of these zero-time activation mixtures were due in part to the rapid initial formation of plasmin by activator as a preliminary step to its further activation. The casein-digestion curves for all samples removed from the activation tubes in this experiment were virtually linear over the 2-hour period of digestion.

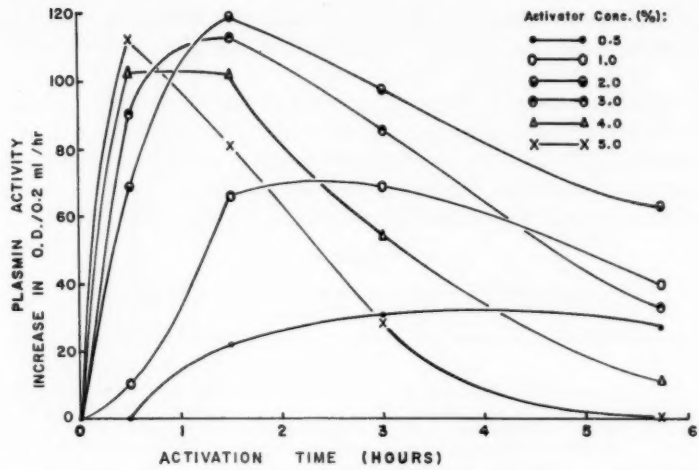


FIG. 6. Effects of activator concentration and incubation time on the activity of generated rat plasmin. Each point on the curves represents the hourly rate of digestion of casein by an aliquot of an activation mixture, containing the concentration of activator shown and maintained at 37° C for the indicated period.

The curves shown in Fig. 6 reveal a number of interesting features with respect to the activation of rat plasminogen. During the initial ½-hour period of incubation, it can be seen that the rate of activation varied directly as the concentration of activator. At high activator concentrations (4.0 and 5.0%, v/v), the plasmin activities developed during this period attained their maximum values, whereas at 0.5% no activity at all had developed and at 1.0 and 2.0% minimum activities were found. In general, the time taken for the development of peak activity bore an inverse relationship to the concentration of activator:

Activator concentration (%, v/v):	0.5	1.0	2.0	3.0	4.0	5.0
Time for peak activity (hours):	4½	2½	1½	1½	½	½

It will be noted that the highest absolute plasmin activities developed in the experiment were at activator concentrations higher than 1.0% (v/v). The use of 3.0% activator at an incubation time of 1½ hours for routine determinations of rat plasmin in physiological work was adopted following consideration of the results of several experiments of this kind. It is evident, however, that other conditions of activator concentration and incubation time may be chosen as "optimal" for the determination of rat plasmin activity.

The results shown in Fig. 6 also demonstrate that the stability of the enzyme as well as its activation is influenced by the concentration of activator present. Following the attainment of maximum plasmin activity at each concentration of activator, it can be seen that inactivation or destruction of the enzyme occurred, and that this inactivation proceeded at a rate which varied directly as the concentration of activator. Thus, at 0.5% (v/v) activator, the highest activity attained by the enzyme was only slightly diminished at the end of 5¾

hours of incubation, whereas at 5.0% (v/v) activator the high rate of inactivation following ½ hour of incubation resulted in complete destruction of the enzyme.

With reference to Fig. 6 it was observed that the relationship between rate of inactivation and activator concentration (1.0 to 5.0%) was one of direct proportionality. When the rate of inactivation at each concentration of activator was determined (measurement of the initial decrease in optical density units over a 2-hour period), it was found (Table I) that the inactivation

TABLE I

The proportionality between activator concentration and rate of inactivation of plasmin

Activator concn. (%, v/v)	Rate of inactivation of plasmin (decrease in O.D. units/hr)*	$\frac{\text{Rate of inactivation}}{\text{Activator concentration}} = k$ (decrease in O.D. units per hr per % activator)
1.0	7.5	7.5
2.0	14.5	7.3
3.0	19.0	6.3
4.0	29.0	7.3
5.0	34.0	6.8

NOTE: Mean k = 7.0 O.D. units per hr per % activator.
*Inactivation rates were determined graphically as the reduction in maximum plasmin activities over a period of 2 hours.

rate expressed as a function of the activator concentration was a constant. The results with 0.5% activator were excluded from these calculations because of the difficulty in obtaining an accurate estimate of the inactivation rate (cf. Fig. 6) at this concentration.

The Proteolysis of Plasminogen during Activation

The observations reported above favor the concept that the activation of plasminogen and the inactivation of generated plasmin in the presence of human euglobulin (activator) are the results of enzyme-catalyzed reactions which cause progressive degradation of the plasminogen molecule. This hypothesis was tested in experiments designed to show that the activation and inactivation processes are accompanied by the proteolysis of plasminogen and plasmin. Rat plasminogen solutions were incubated with activator at 37° C and aliquots (0.7 or 0.5 ml) of the mixture were removed at various times and divided as follows:

(*a*) 0.2 ml was delivered into 3.0 ml of phosphate–saline buffer (pH 8.0) and frozen immediately for subsequent assay of plasmin activity;

(*b*) 0.5 or 0.3 ml was delivered directly into 1.7 *M* perchloric acid to a volume of 3.0 ml for the determination of acid-soluble, tyrosine-like substances absorbing in the ultraviolet at a wave length of 275 mμ. High-speed centrifugation was required for complete precipitation of the reactive proteins.

The results of an experiment of this kind, in which plasminogen was incubated with 5.0% (v/v) activator and samples (0.7 ml) were withdrawn over a period of 3 hours, are shown in Fig. 7. It can be seen that proteolysis occurred

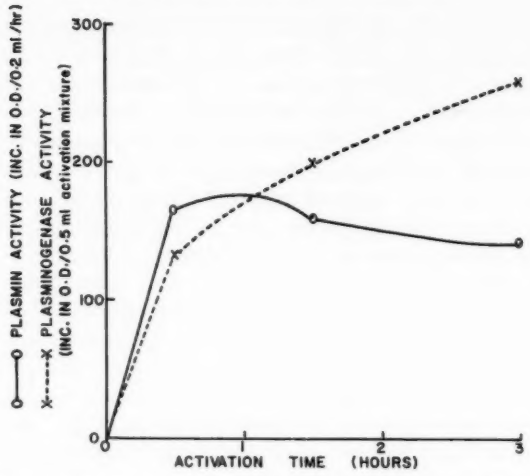


FIG. 7. The proteolysis of plasminogen during activation. Solid lines show the changes in casein-splitting activity of generated plasmin with time of activation. Interrupted lines show the simultaneous increase in acid-soluble, ultraviolet-absorbing substances in the activation mixture. Activator concentration, 5.0% (v/v).

throughout the incubation period, the rate being highest during the initial activation phase and lower during the slow inactivation of the generated plasmin. Other experiments have indicated, however, that the rate of accumulation of tyrosine-like substances in the activation mixture is not readily correlated with the rates of activation and inactivation of plasminogen and plasmin, respectively. Further studies are required to define the significance of proteolysis in these reactions.

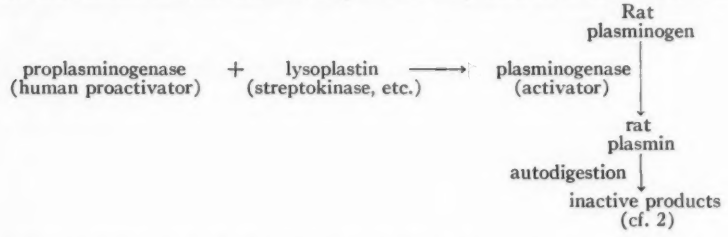
Discussion

The foregoing study was designed primarily to find the optimum conditions required for the determination of rat plasmin activity. Although rat plasma or the euglobulin precipitate therefrom exhibits little or no spontaneous or streptokinase-activated plasmin activity toward casein, the activation of the enzyme by streptokinase-activated human serum euglobulin provided the means for determining plasmin activity in as little as 0.02 ml of rat plasma. The conditions which were found to yield the highest linear rate of casein digestion in these experiments were: activation of the enzyme with 3.0% activator (v/v) for 1½ hours at 37° C and digestion of casein at pH 7.8 to 8.2.

The influence of temperature, incubation time, and activator concentration on the conversion of plasminogen to plasmin strongly suggests that the activation of rat plasminogen is itself an enzymic reaction. Furthermore, the liberation of acid-soluble, ultraviolet-absorbing substances during activation indicates that this reaction is proteolytic and points to the hydrolysis of rat plasminogen as an essential step in its conversion to plasmin. Similar views have been expressed with respect to the activation of plasminogen from other species (12, 16, 21, 37, 38, 39, 45).

The components of the activating system in human serum have not been definitely established. While it is tempting to conceive of activation simply as the proteolytic effect of human plasmin (contained in the streptokinase-activated euglobulin extract) on rat plasminogen, Mullertz and others (2, 20, 38) have stressed the "non-involvement of human plasmin in the process" (2) and presented evidence for the presence of a distinct proactivator–activator system in human serum. Since plasminogens from various species are substrates for human activator, the names proplasminogenase and plasminogenase have been given to proactivator and activator respectively. However, the failure to isolate proactivator from human plasminogen and the indirect nature of the evidence for such an activating system have led several investigators to question these views (40, 48, 49, 50). Although the experiments described above do not attack this problem directly, the absence of caseinolytic activity in control activator solutions, which presumably contain human plasmin, and their effectiveness as protease activators of rat plasminogen tend to support the hypothesis that a distinct activating system is present in human euglobulin extracts.

The activation and inactivation of rat plasmin may thus be viewed as follows:



Observations arising out of the present studies, however, suggest that this scheme may not adequately account for the experimental results. One of the important requirements for the satisfactory determination of plasmin activity is that of linearity in the rate of digestion of casein. As discussed previously with respect to the experiment described in Fig. 5, the concavity of digestion curves following activation at 2° C and 25° C is readily explained by the assumption that activation of the enzyme has occurred during the course of digestion. Presumably, therefore, only when activation of the available enzyme is complete or when the activating system is exhausted or destroyed should linearity of digestion prevail in the presence of excess substrate and for as long as the enzyme (plasmin) is stable. The results described in Fig. 5, however, show that, following the incubation of rat plasminogen with activator at 37° C for various periods of time, the plasmin activity was proportional within limits to the activation time, the digestion rates being constant for all samples. Thus linearity of digestion was observed in the presence of partially activated enzyme-activator samples, which were capable of further activation, as well as in those which exhibited maximum activity. The simplest explanation for this phenomenon is that the activation of plasminogen at 37° C combined with the subsequent effects of dilution, freezing, and thawing destroys

the activator to such an extent that it no longer manifests activity during the digestion of casein. However, since neither a brief activation at 37° C (Fig. 5) nor dilution, freezing, and thawing alone prevented further activation of the enzyme, the possibility must be entertained that other mechanisms are involved which govern the formation of plasmin or influence its activity toward casein.

The results shown in Fig. 6 suggest an alternate explanation for the mechanism of plasminogen activation. As previously stated, zero-time control activation samples, diluted and frozen immediately following the mixture of plasminogen with activator, exhibited considerable casein-splitting activity which varied directly as the activator concentration. This control activity was taken to represent the initial, extremely rapid formation of rat plasmin, the amount being determined by the concentration of activator used. This suggests that the role of activator is simply one of providing the components required in the formation of sufficient plasmin to catalyze the conversion of the remaining plasminogen.

In view of the linearity of casein-digestion curves for all samples during activation, which implies that activation did not occur during digestion owing to the rapid destruction or utilization of activator, this further suggests that the activator itself is not directly involved in the enzymatic activation of plasminogen. The hypothesis which seems best to explain the experimental results is that the activation of plasmin is an autocatalytic process, its rate being predetermined by the initial amount of plasmin formed through the action of activator.

The mechanism of inactivation of plasmin has been given somewhat less attention than the problem of its formation. The most generally accepted theory is that the enzyme undergoes self-destruction by virtue of its own proteolytic powers (37). In the experiments described above it was observed that the stability of rat plasmin was affected not only by the incubation temperature (Figs. 4 and 5) but also by the concentration of activator employed (Fig. 6), the rate of inactivation being directly proportional to the activator concentration (Table I). While these observations point to the direct enzymatic destruction of plasmin by activator they are not inconsistent with the view that inactivation is an autocatalytic process, its rate being determined by the rate of activation of plasminogen which in turn depends upon the concentration of activator employed.

It is proposed, therefore, that activation and inactivation represent progressive stages in the self-degradation of plasminogen, the role of activator being one of determining in some way the initial amount of active enzyme formed. The reaction sequence is thus viewed as follows:

1. Activator + plasminogen $\longrightarrow$ plasmin (stoichiometric ?)
2. Plasminogen $\xrightarrow{\text{plasmin}}$ plasmin
3. Plasmin $\xrightarrow{\text{plasmin}}$ inactive products

Reaction 1 would proceed rapidly to completion at 37° C, and reactions 2 and 3 would occur simultaneously in a given activation mixture.

Acknowledgments

The author wishes to express sincere thanks to Dr. de Guise Vaillancourt and Dr. M. Francoeur for their constant interest and encouragement and to Dr. M. St. Martin, who kindly prepared the fibrinolytic staphylokinase used in early experiments.

References

1. MACFARLANE, R. G. and BIGGS, R. Blood, **3**, 1167 (1948).
2. MULLERTZ, S. Acta Physiol. Scand. **38**, Suppl. 130 (1956).
3. THOMAS, D. P. P. and DINGLE, J. T. M. Acta Rheum. Disease, **14**, 195 (1955).
4. ALBRECHTSEN, O. K. Acta Endocrinol. **23**, 219 (1956).
5. DILLARD, G. H. L. and CHANUTIN, A. Cancer Research, **9**, 665 (1949).
6. BIGGS, R., MACFARLANE, R. G., and PILLING, J. Lancet, **252**, 402 (1947).
7. RATNOFF, O. D. Bull. Johns Hopkins Hosp. **84**, 29 (1949).
8. TAGNON, H. J., LEVENSON, S. M., DAVIDSON, C. S., and TAYLOR, F. H. L. Am. J. Med. Sci. **211**, 88 (1946).
9. SCROGGIE, A. E., JAQUES, L. B., and ROCHA E SILVA, M. Proc. Soc. Exptl. Biol. Med. **66**, 326 (1947).
10. CLIFFTON, E. E. J. Lab. Clin. Med. **39**, 105 (1952).
11. Symposium *on* Proteolytic enzymes and their clinical application. *Edited by* Otto v. St. Whitelock. Ann. N.Y. Acad. Sci. **68**, 1 (1957).
12. CHRISTENSEN, L. R. and MACLEOD, C. M. J. Gen. Physiol. **28**, 559 (1945).
13. YAMAKAWA, S. J. Exptl. Med. **27**, 689 (1918).
14. STEPHAN, R. and WOHL, E. Z. ges. exptl. Med. **24**, 391 (1921).
15. JACKSON, H. D. and MERTZ, E. T. Proc. Soc. Exptl. Biol. Med. **86**, 827 (1954).
16. ALKJAERSIG, N., FLETCHER, A. P., and SHERRY, S. J. Biol. Chem. **233**, 81, 86 (1958).
17. LEWIS, J. H. and FERGUSON, J. H. Am. J. Physiol. **170**, 636 (1952).
18. LEWIS, J. H. and FERGUSON, J. H. Am. J. Physiol. **166**, 594 (1951).
19. KAPLAN, M. H. Proc. Soc. Exptl. Biol. Med. **57**, 40 (1944).
20. MULLERTZ, S. and LASSEN, M. Proc. Soc. Exptl. Biol. Med. **82**, 264 (1953).
21. CHRISTENSEN, L. R. J. Gen. Physiol. **28**, 363 (1945).
22. PERMIN, P. M. Nature, **160**, 571 (1947).
23. TAGNON, H. J. and PALADE, G. E. J. Clin. Invest. **29**, 317 (1950).
24. GOLDHABER, P., CORNMAN, I., and ORMSBEE, R. A. Proc. Soc. Exptl. Biol. Med. **66**, 590 (1947).
25. ASTRUP, T. Biochem. J. **50**, 5 (1951).
26. LEWIS, J. H. and FERGUSON, J. H. J. Clin. Invest. **29**, 1059 (1950).
27. ASTRUP, T. and STERNDORFF, I. Proc. Soc. Exptl. Biol. Med. **81**, 675 (1952).
28. PLOUG, J. and KJELDGAARD, N. O. Arch. Biochem. Biophys. **62**, 500 (1956).
29. SCHULTZ, R. L. and VON KAULLA, K. N. Biochem. J. **68**, 218 (1958).
30. CLIFFTON, E. E. and CANNAMELA, D. A. Blood, **8**, 554 (1953).
31. PALY, S. N. and KLINE, D. L. Yale J. Biol. and Med. **26**, 486 (1954).
32. HEDIN, S. G. J. Physiol. **30**, 195 (1904).
33. SHULMAN, N. R. J. Exptl. Med. **95**, 571, 593 (1952).
34. LEWIS, J. H. and FERGUSON, J. H. J. Clin. Invest. **29**, 486 (1950).
35. JACOBSSEN, K. Scand. J. Clin. and Lab. Invest. **7**, Suppl. 14 (1955).
36. NORMAN, P. S. J. Exptl. Med. **108**, 53 (1958).
37. GEIGER, W. B. J. Immunol. **69**, 597 (1952).
38. TROLL, W. and SHERRY, S. J. Biol. Chem. **213**, 881 (1955).
39. MULLERTZ, S. Biochem. J. **61**, 424 (1955).
40. SHERRY, S. J. Clin. Invest. **33**, 1054 (1954).
41. MILSTONE, H. J. J. Immunol. **42**, 109 (1941).
42. CLIFFTON, E. E. and DOWNIE, R. G. Proc. Soc. Exptl. Biol. Med. **73**, 559 (1950).
43. KLINE, D. L. J. Biol. Chem. **204**, 949 (1953).
44. LASSEN, M. Acta Physiol. Scand. **27**, 371 (1952).
45. LASSEN, M. Biochem. J. **69**, 360 (1958).
46. MULLERTZ, S. Proc. First International Conference on Thrombosis and Embolism, Basel. 1954. p. 75.
47. GOMORI, G. *In* Methods in enzymology. Vol. I. *Edited by* S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York. 1955. p. 138.
48. KLINE, D. L. and FISHMAN, J. B. Ann. N.Y. Acad. Sci. **68**, 25 (1957).
49. SHERRY, S. and ALKJAERSIG, N. Ann. N.Y. Acad. Sci. **68**, 52 (1957).
50. ABLONDI, F. B. and HAGAN, J. H. Proc. Soc. Exptl. Biol. Med. **95**, 195 (1957).

ACETYLCHOLINE IN PERIPLANETA AMERICANA L.

III. ACETYLCHOLINE IN ROACHES TREATED WITH TETRAETHYL PYROPHOSPHATE AND 2,2-BIS(*p*-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE[1]

E. H. COLHOUN

Abstract

The levels of acetylcholine (ACh) in the thoracic nerve cords of cockroaches were increased by the topical application of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) and of tetraethyl pyrophosphate (TEPP), but only TEPP inhibited cholinesterase (ChE). Improvements in the correlation of symptoms, nervous activity, and ACh levels with ChE were obtained when nerve cords were homogenized in saline containing ACh, which prevented further inhibition of ChE by TEPP found to be present in blood and nervous tissue. There was a similarity in the distribution of ACh in thoracic nerve cords of roaches after topical treatment with TEPP and DDT but the physiological properties of the blood revealed differences in the mode of action of the two insecticides. The effects of blood from the poisoned insects on the electrical activity of the isolated nerve cord of roaches are discussed in relation to the penetration of the nerve cord by known neurohumors.

Introduction

Insects poisoned with organophosphorus compounds accumulate acetylcholine (ACh) (1, 2) and there is a correlation between cholinesterase (ChE) inhibition and the levels of ACh (2). As high levels of ACh have been obtained with lethal doses of various inhibitors, it is of considerable importance to establish their origin. Release of ACh by nerve fibers may not account for the substantial increases. In a previous communication it was pointed out that increases in the amount of ACh were obtained in roaches when the central nervous system was electrically silent (3).

As insects live for a considerable time after being given lethal doses of poisons, it is possible that a large proportion of the increase in ACh is attributable to factors associated more with synthesis than with release of ACh by nerve activity. The anomaly of increases of ACh without ChE inhibition in insects treated with DDT (4) might well be resolvable on this basis. The present work deals with ACh physiology in *Periplaneta americana* L. treated with TEPP (tetraethyl pyrophosphate) and DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane).

Materials and Methods

Test Insect

Roaches were obtained from laboratory stocks (5). Male roaches were used except for some blood determinations, for which females were preferred as they gave a larger volume of blood.

Treatment with Inhibitors

TEPP was prepared by Dr. J. R. Robinson of this laboratory. Solutions of TEPP in acetone were applied topically to the dorsal abdomen. The

[1]Manuscript received in original form April 3, 1958, and, as revised, October 24, 1958.
Contribution No. 127, Science Service Laboratory, Canada Department of Agriculture, London, Ontario.

minimum lethal doses determined by probit analysis were 5 μg per male roach and 7.5 μg per female roach. DDT supplied by Naugatuck Chemicals was dissolved in dioxane (6) and 50 μg was applied to the sternum between the front coxae; this dose was lethal to roaches kept below 25° C. The dose was not lethal to roaches kept at 35° C, but became lethal after the roaches were transferred to 15° C.

Extraction of Nerve Tissue and Blood and Assay for Acetylcholine

Thoracic nerve cords of roaches were removed by dissection (7). ACh determinations were carried out in duplicate on pooled samples of five thoracic nerve cords for each experiment. Each experiment was replicated at least four times. Total dissection time for five cords averaged 2 minutes. The cords were weighed and placed in 5 ml of boiling HCl-acidified frog Ringer's solution, pH 3.9, for 30 seconds, cooled, and homogenized in the same 5 ml of Ringer's solution containing 1% trichloroacetic acid. The frog Ringer's solution contained NaCl 7 g, KCl 0.14 g, $CaCl_2$ 0.12 g per liter. The homogenate was centrifuged, the supernatant decanted and brought, with constant stirring, to pH 6.0 with 0.3 *N* NaOH. The volume was made up to 10 ml with unbuffered frog Ringer's solution, and divided into two 5-ml aliquots; one of these was made alkaline with 0.3 *N* NaOH, heated to destroy ACh, then neutralized with 0.3 *N* HCl.

For determinations of 'free' and 'bound' ACh (8) 15 thoracic nerve cords were pooled and homogenized in ice-cold eserinized saline. This saline differed from frog Ringer's solution in that the amount of NaCl was increased to give a molarity of 0.314. The homogenate was centrifuged at 18° F in a Spinco centrifuge at 50,000 *g* for 60 minutes. The supernatant was decanted and divided into aliquots as described above. The residue was placed in 5 ml of boiling HCl-acidified frog Ringer's solution at pH 3.9, homogenized, and the supernatant treated as above.

For determinations of the ACh content of ganglia and connectives (nerve fibers between ganglia) of the thoracic nerve cord, the cords were dissected out and placed on saline-moistened filter paper and then separated into ganglia and connectives with fine scissors. Care was taken to avoid squeezing.

Blood was obtained from groups of 100 roaches by cutting their antennae and exerting gentle pressure on the thorax and abdomen, thus allowing blood to drip out of the antennae (6). The blood was collected on a watch glass kept over dry ice. One hundred males yielded 0.4 ml. A similar number of females yielded up to 0.8 ml. Blood for ACh assays was taken up in 5 ml of frog Ringer's solution containing 1% trichloroacetic acid, centrifuged, and the supernatant treated as previously described. Blood for electrophysiological studies was thawed, centrifuged, and the straw-colored serum was drawn off and frozen until required. Roaches treated with DDT were kept at 35° C for 20 hours, then at 15° C for 2 hours, and were bled.

The ACh content of perfusates of whole isolated ventral cords was determined by dissecting out cords, then placing them in groups of five in 2 ml of

frog Ringer's solution. The perfusate was collected at intervals and tested for ACh on the *rectus abdominis* muscle of the frog.

ACh assays were carried out using the *rectus abdominis* muscle of the frog (*Rana pipiens*). Blood constituents were also tested using the heart of *Venus mercenaria* (7). Corrections were made for sensitizing substances (9). The ACh values reported here are given in terms of acetylcholine bromide. The identity of ACh was also confirmed by hydrolysis by ChE and alkali and by the antagonistic effect of atropine. Furthermore, ACh is the only choline ester found in the nerve cord of roaches (10). For assays of ACh, buffered frog Ringer's solution was used which contained NaCl 7 g, KCl 0.14 g, $CaCl_2$ 0.12 g per liter, and $NaHCO_3$ to pH 7.3.

Working Determinations of Cholinesterase Activity

ChE activity was measured manometrically at 25° C. The main compartment contained 2 ml of $NaHCO_3$ solution, final concentration 0.025 *M*, and 0.5 ml of enzyme preparation. The side arm contained 0.2 ml ACh bromide, final concentration 0.01 *M*. The atmosphere was saturated with 5% CO_2 in N_2. Readings were made at 10-minute intervals. Corrections were made for endogenous acid formation of the enzyme preparation in the absence of substrate.

ChE activity was determined in duplicate for each experiment, using five thoracic nerve cords homogenized in 2 ml of ice-cold 0.9% saline. At least two experiments were carried out in each case. When ACh was used in excess to protect against further inhibition of ChE during homogenization, the cords were homogenized in saline containing 0.3% ACh bromide and the homogenate was frozen until required. In this case a compensatory reduction was made in the ACh concentration in the side arm; 4.5 mg in 0.2 ml was used.

Electrophysiological Studies

To test blood constituents the nervous activity of the isolated nerve cord of the roach was recorded. The cord was placed within depressions in a wax block so that only the thoracic ganglia and sixth abdominal ganglion were bathed in the test fluid and recordings were made between the second and third abdominal ganglia. When recording electrical activity of the ventral cord of the intact roach, a portion of ventral abdominal cuticle was cut away and chlorided silver electrodes were inserted around the cord. Electrical activity was recorded with a Tektronix 512 oscilloscope, Tektronix Incorporated, Portland, Oregon, and an Ampex 601 tape recorder, Ampex Corporation, Redwood City, California.

Isolated Heart Preparation

The abdomen was slit dorsally and eviscerated. The testicles and accessory glands were removed. The abdomen was cut off, pinned open to a wax block with the ventral side uppermost, and perfused with Ringer's solution containing the test substance.

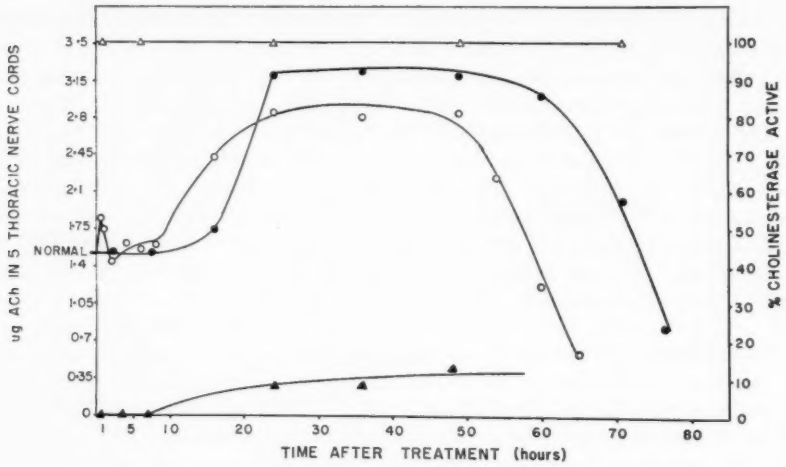


FIG. 1. Acetylcholine levels and cholinesterase inhibition in roach thoracic nerve cord after treatment with TEPP and DDT. ○ ACh content after TEPP, ● ACh content after DDT, ▲ ChE inhibition after TEPP, △ ChE inhibition after DDT. Experiments for ACh levels were carried out at 20 to 22° C.

Results

ACh Levels in Roach Thoracic Nerve Cord

The ACh contents of thoracic nerve cord after topical treatment of roaches with TEPP and DDT are shown in Fig. 1. Both poisons increased the ACh content of the nerve cord and the results with DDT confirm previous findings (4), although the increase found in the present work was less than that reported by Tobias *et al.* (4). That both poisons lead to an increase in ACh is suggestive of a common factor in the toxic action of TEPP and DDT, yet only TEPP inhibited ChE (Fig. 1). This common feature of a strikingly high increase in ACh appeared only after the roaches had been prostrate for some time. The eventual decline in ACh levels to below normal was previously reported in the action of organophosphorus compounds on flies by Smallman and Fisher (2), who pointed out that the decrease coincided with the recovery of ChE activity. In roaches, however, the decrease seems to coincide more with necrosis which appeared earlier in TEPP-poisoned than in DDT-poisoned roaches. Necrosis was retarded when roaches treated with these compounds were kept at 15° C; the decrease in ACh levels was also retarded. In the instance of DDT the rate of decline of ACh can be seen by comparing Table I with Fig. 1.

In the early stages of poisoning there are striking differences in the ACh content of the thoracic nerve cord of roaches poisoned with TEPP and with DDT (Fig. 1). In the more prolonged hyperactive phase of DDT intoxication there was no increase in ACh content which rose rapidly within the 1st hour after TEPP treatment. The difference does not appear to be related to the

TABLE I

The ACh content of thoracic nerve cord of roaches treated with DDT after temperature change*

Temperature, ° C	Time, hours	Control, μg ACh in 5 cords	DDT treatment, μg ACh in 5 cords
35	20	1.95	2.26
15	2	1.68	1.88
15	26	1.44	2.60
15	120	1.46	2.63

*Roaches were placed at 35° C for 20 hours, then transferred to 15° C where they became hyperactive and prostrate. Nerve cords were removed at the designated times.

rapid prostration of the TEPP-treated roaches for prostration can be quickly induced by DDT if the roaches are first kept at 35° C for 20 hours and then cooled at 15° C (6). The roaches became prostrate within one-half hour. No increase in ACh levels followed this treatment (Table I), indeed, the levels of ACh obtained were lower than those found at 35° C, but this reduction is due to the effects of temperature, for it is found in normal roaches (7). After 24 hours at 15° C the concentration of ACh rose to a high level similar to that shown for DDT (Fig. 1).

Cholinesterase Activity and Acetylcholine Levels

The results for ChE activity without substrate protection (ACh) during homogenization are illustrated in Fig. 1. It is apparent that immediate and complete inhibition was obtained with TEPP. This observation is similar to that found by Smallman and Fisher for flies (2). The first indication of incorrect in vivo ChE results was obtained when nerve cords of roaches treated with TEPP were removed and homogenized and their ACh content compared with that of cords boiled immediately after dissection. Inclusion of eserine in the homogenization fluid was as effective as boiling. The results in Table II show a loss of ACh in the non-eserinized homogenate of cords suggesting that some ChE was still uninhibited. With DDT no ACh was found in the non-eserinized homogenate. The validity of the results for ChE inhibition as shown in Fig. 1 was, therefore, questionable and it remained possible that the TEPP, which was still free in the nerve tissue, might inhibit ChE in the course of homogenization. This possibility was confirmed by homogenizing together nerve cords of normal roaches and those of treated roaches and by assaying the resultant ChE inhibition (Table III). Significant amounts of free TEPP were found up to 7 hours after administration of TEPP. Using this method it was possible to estimate the amount of 'free TEPP' in nerve cords of treated roaches (Table III and Fig. 2).

After these results were obtained, experiments were performed to determine the probable in vivo inhibition of ChE of roach nerve cord, using the observation that inhibition of fly-head ChE by organophosphorus compounds could be determined more precisely by the addition of excess ACh during homogenization (11). The validity of the technique is outlined in the experiments

TABLE II

A comparison of the ACh content of roach thoracic cords after treatment with TEPP when homogenized with eserine sulphate

Time after treatment, hours	No. of determinations	μg ACh in boiled cords		μg ACh in eserinized homogenate		μg ACh in non-eserinized homogenate		Per cent loss of ACh in non-eserinized homogenate
		Mean	Range	Mean	Range	Mean	Range	
0.5	3	1.80	(1.60–2.00)	1.70	(1.56–1.82)	1.30	(1.26–1.33)	23.5
2	4	1.21	(1.00–1.31)	1.10	(1.00–1.22)	1.00	(0.90–1.20)	9.1
7	5	1.67	(1.58–1.80)	1.66	(1.55–1.71)	1.36	(1.00–1.23)	18.1
16	4	2.31	(2.17–2.50)	2.22	(2.10–2.34)	1.42	(1.40–1.48)	36.0
24	10	2.60	(2.40–2.94)	2.60	(2.45–3.00)	1.66	(1.45–1.81)	36.2
36	3	2.87	(2.35–3.10)	2.74	(2.45–3.21)	1.50	(1.22–1.93)	45.3
48	4	2.90	(2.60–3.20)	2.91	(2.61–3.50)	1.30	(0.9 –1.53)	55.5

TABLE III

Free TEPP in roach thoracic nerve cord determined by cholinesterase inhibition of normal cords when combined with cords from treated roaches

Time after treatment, hours	b_{30}*		Per cent ChE inhibited	μg TEPP+ in 5 treated thoracic cords
	Normal thoracic cords	Normal + treated thoracic cords		
0.5	80.9±11.1	0	100	0.029
2		25.2±2.1‡	69	0.0035
7		60.6±3.1	25	0.003
24		78.0±3.1	0	0

*b_{30} refers to the μl CO_2 evolved during 30 minutes of enzyme hydrolysis according to the method of Augustinnson, 1948.
†Extrapolated from Fig. 2.
‡Standard deviation.

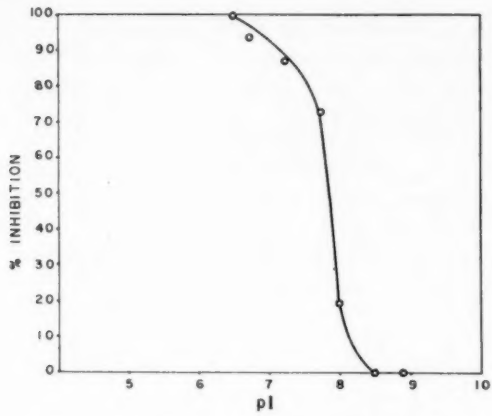


FIG. 2. Inhibition by TEPP of ChE of the roach thoracic nerve cord.

of Burgen (12) and Aldridge (13). The results given in Table IV show that ChE was not fully inhibited in vivo until 30 minutes after treatment. These results show some correlation with the loss of endogenous ACh during homogenization (Table II). The loss of ACh at 7 hours is difficult to explain for at this time ChE was completely inhibited (Table IV). The more accurate estimate of ChE inhibition obtained by these methods invalidates the data given in Fig. 1 for ChE inhibition during early TEPP poisoning, but the evidence for ChE inhibition from 7 hours onwards was not influenced by the addition of excess substrate. Significantly, no 'free' TEPP was found beyond 7 hours. These results suggest that it is possible to obtain a correlation between symptoms, nerve activity, and the ACh increases during early TEPP poisoning, although difficulties are evident from the ACh curve (Fig. 1) up to 7 hours. Here, an initial rise of ACh was found at 30 minutes which fell to below normal at 2 hours. This problem is, at present, under investigation particularly as ACh was found in roach blood at 2 hours (3). This latter fact is specific evidence that TEPP treatment resulted in free ACh.

The Acetylcholine Content of Thoracic Nerve Cord of Roaches at Late TEPP and DDT Treatment

It is evident from Fig. 1 that high levels of ACh were found in the presence of uninhibited ChE in the late stages after treatment with TEPP and DDT. Is there then a similarity between the in vivo forms of ACh in the nerve cords of roaches poisoned with DDT and TEPP? The first evidence of such a similarity was obtained in the distribution of ACh in the thoracic ganglia and connectives. The results given in Table V show that the ratio of levels of ACh in connectives to ganglia was 1:3, and this same ratio was obtained after treatment with TEPP and with DDT. The ACh content of the connectives of TEPP-treated roaches was slightly higher than that found for DDT.

TABLE IV

Effect of addition of ACh at homogenization on ChE inhibition of roach thoracic nerve cord after topical treatment with TEPP

Time after treatment, hours	b_{30}			Per cent protection by excess ACh in homogenate	Per cent ACh level in thoracic cords of treated roaches	Gross electrical activity of ventral nerve cord *in situ*
	Normal thoracic cords	Thoracic cords from treated roaches	Thoracic cords from treated roaches + excess ACh			
0.25	69.6±4.9	0	40.8±3.1*	58	+8	Intense
0.5 (Hyperactive)		0	34.3±4.1	49	+20	Intense
0.5 (In-co-ordinated)		0	19.1±5.0	27	—	Intense
1		0	0	0	+13	
2 (Prostrate, twitching)		0	0	0	−8	Reduced
7 (Prostrate, twitching)		0	0	0	+6	Reduced
24 (Prostrate, motionless)		7.4 (7.0–7.8)	8.3 (8.0–8.5)	0	+90	None
48 (Prostrate, motionless)		13.0 (11.0–15.0)	11.0 (10.0–12.0)	0	+93	None

*Standard deviation.

TABLE V

Comparison of the ACh content of ganglia and connectives of roach thoracic nerve cord at 24 hours after topical treatment with TEPP and DDT

Treatment	μg ACh on* ganglia + connectives	μg ACh in ganglia	μg ACh in connectives	Per cent ACh in ganglia	Per cent ACh in connectives
Normal	2.67±0.17	1.78±0.12	0.89±0.05†	66.6	33.4
TEPP	5.47±0.53	3.32±0.34	2.15±0.19	60.7	39.3
DDT	5.77±0.63	3.66±0.43	2.11±0.21	63.4	36.6

*Ganglia and connectives of 10 thoracic cords per experiment.
†Standard deviation.

This evidence is in contradiction to the data of Tobias *et al.* (4), who found that ACh increases were obtained mainly in connectives, and stated that this increase was free ACh. The similarity of these results prompted an investigation into the amounts of ACh that would be found in supernatants and residues of homogenates of cords of DDT- and TEPP-treated roaches, after high-speed centrifugation. As shown in Table VI, agreement was obtained between TEPP and DDT in the amounts of ACh obtained in each fraction of the centrifugate. If the above normal ACh had been 'free' in vivo in TEPP-treated nerve cords, one would expect that more ACh would be found in the supernatant. These results merely point to a similarity between ACh in TEPP- and DDT-treated nerve cords and it is emphasized that these results do not indicate the probable in vivo 'free' and 'bound' ACh ratio, for the determination of which no satisfactory method has yet been devised (14). The 'freeing' of ACh from roach nerve tissue can be achieved by a combination of physical methods (8) and thus in this way it differs from mammalian nerve tissue. The results given in Table VI can be altered by the severity of homogenization, the pH of the extraction medium, osmotic pressure, and ion balance; but in each instance no difference has yet been found between the effects induced by DDT and by TEPP. This problem will be discussed more fully in a separate publication with reference to the intracellular distribution of choline acetylase.

Evidence of a dissimilarity in the ACh content of nerve cords of roaches treated with TEPP and DDT was obtained at 24 hours by placing isolated nerve cords in Ringer's solution. The perfusate was assayed for ACh and it was found that the perfusate of nerve cords of TEPP-treated roaches contained 0.09–0.10 μg of ACh per ml of perfusate. This amount is negligible when compared with the total amount of ACh in the nerve cord, but no ACh whatever was found in the perfusate of nerve cords of DDT-treated or normal roaches. Free ACh evidently escaped from some part of the nerve cord into the perfusate. In this respect nerve cords from TEPP-treated roaches differ from those of normal and DDT-treated roaches. The origin of the ACh in the perfusate is unknown. It was not related to nerve activity for at this time the nerve cord was electrically silent.

TABLE VI

ACh in supernatants and residues of homogenates of roach thoracic nerve cord at 24 hours after topical treatment with TEPP and DDT*

Treatment	μg total ACh (15 cords)	μg supernatant ACh	μg residue ACh	Per cent ACh in supernatant
Normal	4.68±0.38	2.83±0.21†	1.83±0.22	60.9
TEPP	7.62±0.61	6.32±0.51	1.30±0.23	82.9
DDT	8.32±0.45	6.92±0.52	1.41±0.11	83.1

*Homogenized at pH 5.0 to 5.2.
†Standard deviation.

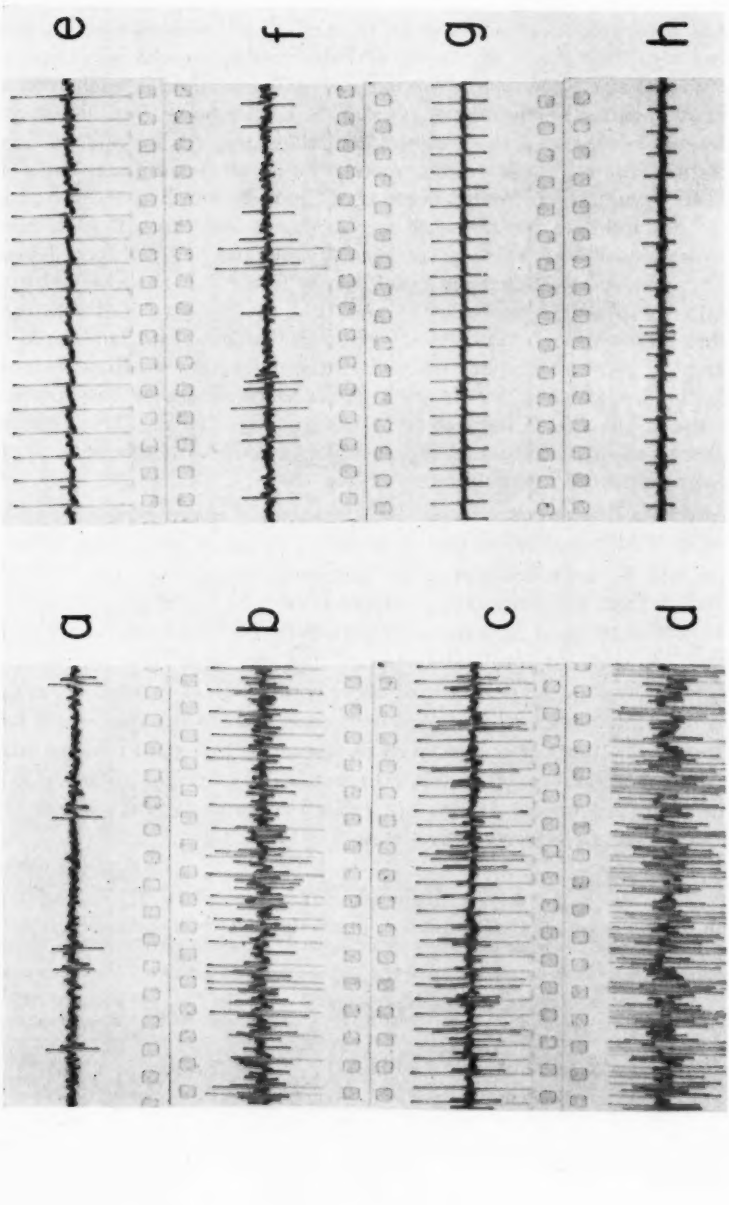


FIG. 3. Electrical activity of the isolated roach nerve cord. (*a*) Normal cord; (*b*, *e*) blood from DDT-treated roaches; (*c*) blood from TEPP-treated roaches; (*d*) corpus cardiacum extract. Blood from DDT-treated roaches on (*f*, *g*) the sixth abdominal ganglion and (*h*) the thoracic ganglia.

Blood Physiology of DDT- and TEPP-treated Roaches

During the course of the foregoing experiments results were obtained on blood physiology which pointed to specific differences in the mode of action of TEPP and DDT. These results may help to explain the cause of high accumulations of ACh in the central nervous system of roaches after treatment with DDT and TEPP. It was found that the blood of roaches treated with TEPP contained ACh in the amount of 1.5 μg/ml at 2 hours and 0.5 μg/ml at 7 hours. In contrast the blood of DDT-treated roaches contained no ACh, either at 2 hours, 7 hours, or 16 hours after treatment, or when roaches were subjected to a temperature change causing quick prostration. The ACh content of nerve cords of DDT-treated roaches remained at the normal level (Fig. 1) at the time the blood was extracted, whereas the ACh content was abnormal in TEPP-treated roaches. The blood physiology experiments were extended to determine whether the blood of DDT-treated roaches contained substances (15, 6) not present in the blood of TEPP-treated roaches. It was found that blood from roaches treated with both DDT and TEPP increased the electrical activity of the roach isolated nerve cord (Fig. 3*b*, *c*). In the case of blood from TEPP-treated roaches it was subsequently determined that residual TEPP, 3.8 μg at 2 hours and 2.4 μg at 7 hours, was present in 0.5 ml blood; it was difficult to separate the effect shown in Fig. 3*c* from the effect of TEPP alone. It was concluded that blood from DDT-treated roaches contained a substance not at present demonstrable in blood from TEPP-treated roaches.

The identity of the substance in the blood of DDT-treated roaches is unknown and it may be non polar for it penetrated the sixth abdominal ganglion as shown by the electrical activity in Fig. 3*f*, *g*, and also the thoracic ganglia (Fig. 3*h*). The effect on the isolated nerve cord was variable as shown in Fig. 3*b*, 3*e*. A number of compounds were tried in order to simulate the effect of DDT-treated blood on the isolated nerve cord. These were adrenaline, noradrenaline, ACh, 5-hydroxytryptamine, and gamma-aminobutyric acid. At concentrations of 10^{-2} *M* to 10^{-8} *M* these substances were ineffective presumably because polar substances do not penetrate undesheathed insect nerve cord (16, 17). It was found that extracts of the corpus cardiacum gland excited the isolated nerve cord (Fig. 3*d*) in a manner similar to the electrically observed excitation of the cords (Fig. 3*b*) by blood from DDT-treated roaches. Also, Chefurka and Colhoun (unpublished data) found by partition chromatography that blood extracted from DDT-poisoned roaches contained abnormally high amounts of this substance previously demonstrated in the corpus cardiacum (18, 19, 20). The substance is not the adrenaline or noradrenaline demonstrated in insects by Ostlund (21), but has been termed 'insect adrenaline' (22).

Discussion

The effect of topically applied TEPP and DDT on the ACh levels of the roach thoracic nerve cord has been examined. Both insecticides caused an increase in ACh content attributed in the case of the organophosphate to an

inhibition of ChE. But as DDT is not an anticholinesterase an alternate explanation of the increase, previously recorded by Tobias *et al.* (4), must be found.

Even in the case of TEPP, poisoning factors other than ChE inhibition may be involved, for two phases of ACh increase are found, the first during hyperactivity, the second and much higher level when the roaches are prostrate. The device of protecting ChE by excess substrate (11) revealed a general correlation between ACh levels, ChE inhibition, and symptoms during the early stages of TEPP poisoning but provided no explanation of the reduction at 2 hours of ACh level to below normal, though at that time ChE was completely inhibited, nor of the reappearance of ChE during the later stages of TEPP poisoning at a time of higher ACh levels.

The initial rise in ACh levels appearing within an hour of treatment with TEPP is probably associated with the intense nervous activity revealed on the oscilloscope. The second much higher rise, similar to that found in DDT-poisoned roaches, is evidence that ACh synthesis proceeds even though the insect is prostrate and its central nervous system exhibits little electrical activity. At this stage in both TEPP- and DDT-treated insects the ratio of the ACh content of ganglia and connectives of thoracic nerve cord is similar to that of the cord of the untreated roach (Table V). This result indicates a parallel increase of ACh in ganglia and connectives and, since the distribution ratio is not altered, provides evidence of 'bound' ACh. Further evidence of the similarity of the ACh increases in TEPP- and DDT-treated roaches is found in the results (Table VI) in supernatant and residue ACh where, under similar experimental conditions, almost the same values are obtained for each treatment. These results do not show the difference which might have been expected if the high ACh increase in TEPP-treated roaches had represented free ACh. The loss of endogenous ACh from non-eserinized homogenates of TEPP- and DDT-poisoned nerve cords indicates that it is unlikely that free ACh existed in vivo to any great extent. The eventual disappearance of ACh from the cord of poisoned insects may be the consequence of necrosis and the liberation and the hydrolysis of 'bound' ACh since the level fell below normal. There is no evidence that the particular hydrolysis is specifically dependent upon reactivation of ChE as suggested by Smallman and Fisher (2), because the ACh of the thoracic cords of roaches immobilized by carbon dioxide eventually fell to a level not detectable by frog muscle assay. Moreover, there is a similar fall in the ACh level of DDT-poisoned nerve cord, though the activity of ChE is unaffected. This finding is explained if the ChE and its substrate ACh are spatially separated in some particular manner, an explanation which also accounts for the existence of ACh in TEPP-poisoned roaches in the presence of 8 to 13% uninhibited ChE. ACh in a form not available in vivo as substrate for ChE may be termed 'bound' ACh. Some free ACh is found in roach thoracic cord at late TEPP poisoning as shown by its appearance in perfusates. This observation indicates that not all of the increase in TEPP-treated roaches is 'bound' ACh. Apart

from this anomaly the present findings are consistent with the hypothesis that ACh increases in late TEPP and DDT poisoning are the result of ACh synthesis in a form not available to ChE. This would resolve the anomaly of an ACh increase in DDT-treated roaches and helps to explain the high rise in roaches treated with TEPP, an observation difficult to reconcile with nervous activity. The data of Smallman and Fisher (2) for ACh increases in flies after treatment with a number of organophosphorus compounds, show rates of increase different for each compound. These compounds may be exerting an effect of ACh synthesis distinct from nervous activity and some evidence of this is found in the work of Winteringham *et al.* (23). There is also evidence that DDT may exert an effect upon ACh synthesis (24). It should also be recognized that ACh increases occurring in insects for many hours after immobilization may be peculiar to organisms "living" without the same dependency on vital systems as vertebrates, for in the latter death is rapid if either heart, nervous system, or respiration is interfered with. Body temperature falls and tissues undergo changes without their supply of oxygen and blood. Although these systems are interfered with in poisoned insects the tissues apparently do not degenerate for some time. Death in insects may be more a matter of individual tissue degeneration.

Distinct differences are found for the mode of action of TEPP and DDT at early poisoning. In the initial stages of TEPP poisoning ACh is found in roach blood but none after treatment with DDT. The presence of ACh in roach blood is firm evidence that free ACh can be released by insect nerve tissue for these releases appear during hyperactivity and corresponding intense nervous activity is revealed by the oscilloscope. This is additional evidence of a functional role for ACh in the insect nervous system. Although ACh is found in the blood of TEPP-treated roaches there is no evidence that the ACh is released by the central nervous system for Colhoun (25) showed that ACh did not escape from eserinized roach nerve cord *in situ*.

Blood from DDT-treated roaches exerted biological effects which could not be demonstrated with blood bled from TEPP-treated roaches because of the presence of TEPP in the blood. These effects were demonstrated as an increase in the rate of heart beat of the roach isolated-heart preparation and stimulation of the nervous activity of the roach isolated-nerve cord. Partition chromatography of this blood* revealed the presence of high titers of various substances, two of which have been identified as hormones of the corpus cardiacum and corpus allatum. Only the corpus cardiacum hormone simulated the effect of blood on the nerve cord and heart preparations. Since Sternburg *et al.* (15, 6) have demonstrated the paralytic effect of blood from DDT-treated roaches when injected into other insects, it is interesting to speculate that this effect may in part be due to the corpus cardiacum hormone. Insects undergo intense stress when treated with nerve poisons, as seen by the resulting symptoms, and undoubtedly many substances, including those under nervous control, find their way into the circulatory system. A problem

*Chefurka and Colhoun E. H. Unpublished data.

yet to be answered in DDT poisoning is the mechanism of prostration. This may be due to an effect of DDT on peripheral fibers which indirectly results in an accumulation of substances in blood, for in TEPP-treated roaches the ACh which is found in blood is an indicator of events occurring in the nervous system during the initial stages of poisoning.

Acknowledgments

Thanks are due to Miss S. Bushila for technical assistance and to Dr. R. D. O'Brien for helpful discussion during the cholinesterase determinations.

References

1. LEWIS, S. E. and FOWLER, K. S. Nature, **178**, 919 (1956).
2. SMALLMAN, B. N. and FISHER, R. W. Can. J. Biochem. and Physiol. **36**, 575 (1958).
3. COLHOUN, E. H. Science, **127**, 24 (1958).
4. TOBIAS, J. M., KOLLROSS, J. J., and SAVIT, J. J. Cellular Comp. Physiol. **28**, 159 (1946).
5. FISHER, R. W. and JURSIC, F. Can. Entomologist, **90**, 1 (1958).
6. STERNBURG, J. and KEARNS, C. W. Science, **116**, 144 (1952).
7. COLHOUN, E. H. J. Insect Physiol. **2**, 108 (1958).
8. COLHOUN, E. H. Nature, **181**, 490 (1958).
9. FELDBERG, W. E. J. Physiol. **103**, 367 (1945).
10. CHANG, S. C. and KEARNS, C. W. 3rd Meeting Entomol. Soc. America, Cincinatti, Ohio. 1955.
11. VAN ASPEREN, K. Nature, **181**, 355 (1958).
12. BURGEN, A. S. V. Brit. J. Pharmacol. **4**, 219 (1949).
13. ALDRIDGE, W. N. Chem. & Ind. (London), 473 (1954).
14. BRODKIN, E. and ELLIOTT, K. A. C. Am. J. Physiol. **173**, 437 (1953).
15. STERNBURG, J., CHANG, C. S., and KEARNS, C. W. Federation Proc. **16**, 124 (1957).
16. HOYLE, G. J. Exptl. Biol. **30**, 121 (1953).
17. TWAROG, B. M. and ROEDER, K. D. Ann. Entomol. Soc. Am. **50**, 231 (1957).
18. CAMERON, M. L. Nature, **172**, 349 (1953).
19. UNGER, H. Naturwissenschaften, **43**, 66 (1956).
20. UNGER, H. Biol. Zentr. **76**, 204 (1957).
21. OSTLUND, E. Acta Physiol. Scand. **31**, 1 (1954).
22. WIGGLESWORTH, V. B. Pubbl. staz. zool. Napoli, **24**, 51 (1954).
23. WINTERINGHAM, F. P. W., HARRISON, A., MCKAY, M. A., and WEATHERLEY, A. Biochem. J. **66**, 49P (1957).
24. LEWIS, S. E. Nature, **172**, 1004 (1953).
25. COLHOUN, E. H. J. Insect Physiol. **2**, 117 (1958).

THE EFFECT OF SYSTEMATIC TRAINING ON PLASMA ELECTROLYTES, HAEMATOCRIT VALUE, AND BLOOD SUGAR IN THOROUGHBRED RACE HORSES[1]

FRANK A. SRÉTER[2]

Abstract

Experiments were carried out on regularly ridden horses and thoroughbred race horses following a 10- to 40-minute training period for both groups and following 1- to 3-minute racing in the latter group. An increase was observed in the concentration of calcium in serum and in the concentration of potassium, inorganic phosphorus, and iron in plasma. In the trained race horses a considerable increase was noted in the haematocrit (Hct) value and in the haemoglobin (Hb) concentration of the blood, average values after the race being 23 g% and 66% respectively. In regularly ridden horses and in thoroughbred horses which were not in training, similar muscular exercise did not produce such a noticeable increase in the Hb concentration. Sixteen to twenty-four hours after a race or exercise a transitory, quite pronounced hyperbilirubinaemia of 3 mg% was observed. A large part of the mobilized red cells was not restored, but disintegrated, causing a transitory hyperbilirubinaemia. In blood samples drawn immediately after the race, the average fragility test value of the red cells increased, haemolysis occurring in 0.63% NaCl solution. Blood samples taken from thoroughbred horses after exercise and racing were hyperglycaemic while the blood sugar levels in exercised, regularly ridden horses were not significantly increased and even decreased following exhaustive galloping.

These experiments support the hypothesis that the hyperglycaemia is the result of an emotional factor influencing a conditioned nervous response. Thus race horses differ from untrained, regularly ridden horses in that the former show reflex red cell mobilization and hyperglycaemia while the latter do not.

Introduction

Thirty years ago Izquierdo and Cannon (1) demonstrated that the emotional factor has a decisive effect in the determination of the red cell count which may increase by as much as 20 to 30% in cats on excitement, as, for example, sudden sight of a barking dog. It seemed to them that the spleen played an important role as a reservoir of blood cells. Dill *et al.* (2) suggested that the changes in the blood are due simply to loss of water from the blood during work, since in non-fatigued subjects the changes consist chiefly of those related to transfer of fluid carrying diffusible ions from the blood to the tissues with the exception of a disproportionate increase in inorganic phosphate and serum calcium.

Many of the publications related to the changes occurring through training have been carried out on athletes and university students while others made use of animals such as dogs or rats as experimental subjects. Experiments on human subjects have not produced unanimous agreement that the training period can cause a permanent change, demonstrable during rest, in the component elements of the blood or in the haemoglobin (Hb) concentration. Steinhaus (3), in his comprehensive report, observed that training slightly increases the Hb concentration, the total mass of corpuscles,

[1]Manuscript received September 2, 1958.
Contribution from the Department of Physiology, Faculty of Animal Husbandry, The University of Agriculture, Budapest, Hungary.
[2]Present address: Department of Anatomy, University of British Columbia, Vancouver, B.C.

and the total blood volume. He found that the bone marrow of regularly exercised dogs is a darker red than that of unexercised litter mates, indicating an increase in bone marrow activity. Scheunert and Krzywanek (4) reported in experiments on horses that a 5-minute trot resulted in an increase in haematocrit (Hct) value from 26.6 to 37.6%. This increase was closely related to the intensity of the work performed. In their opinion, an isotonic, protein-containing liquid leaves the blood stream during the work period, making room for red cells originating in the blood reservoir. The haematocrit value of blood from the extirpated spleen of this horse was 61% as compared with the venous haematocrit level of 24%. Recently Irvine (5) published a report on increased Hb level and Hct value after excitement or exercise on race horses. Brenon (6) also reported a large increase in the red cell count, Hb concentration, and Hct value determinations in four horses after the race. The purpose of the experiments reported here was to determine what changes occur in blood components in systematically trained or exercised race horses in comparison with horses which are regularly ridden but not systematically trained. The experiments were designed to show whether or not phenomena similar to those reported in the literature for trained athletes might be observed in race horses. Changes in blood sugar levels as well as in plasma electrolytes were measured.

Methods

The experiments were performed on 8 adult, well-developed, and regularly ridden horses (thoroughbred or half-bred) and on 34 thoroughbred galloping race horses. Changes relating to the intensity of exercise were determined by repeating experiments on regularly ridden horses in such a way that on the first occasion the horses were exercised for 20 to 25 minutes at an easy or light gallop and on the second occasion, 1 week later, for 30 to 40 minutes at a forced gallop. In the latter case the horses appeared fatigued. Two of the race horses were new at the start of training whereas the remainder had participated in several races that season. The experiments on race horses can be divided into two groups: those carried only through the training period and those carried through three races. In the first race the distance was 2000 meters (1.242 miles) and the time of the winning horse, 3 minutes 14.4 seconds, the second race was 1000 meters (0.621 miles) and the time, 1 minute 1.4 seconds; the third race was 1600 meters (0.93 miles) and the time, 1 minute 44.4 seconds. The individual horses had participated in as few as 6 or as many as 23 races during their careers as racing horses. The relationship between the results and the condition of the horses will be discussed in detail in a later paper.

The first blood samples from the race horses were drawn in the horse box before exercising. The second samples were drawn at the race track immediately after 10 to 15 minutes of training exercise. On a few occasions, some samples were taken at different periods of time after the exercise or race in order to give a more complete picture of the changes occurring during recovery. During the racing season, the committee in charge of race horses

TABLE I

The influence of exercise on various blood constituents

	Thoroughbred galloping race horses				Regularly ridden horses			
	In race		In training		Light gallop		Forced gallop	
	Resting*	After exercise	Resting	After exercise	Resting	After exercise	Resting	After exercise
Plasma Na, meq/l.	136.6 ±0.58	140.8 ±1.00	136.8 ±0.83	140.7 ±1.40				
Plasma K, meq/l.	3.88±0.03	4.28±0.06†	3.80±0.05	4.10±0.06†				
Serum Ca, meq/l.	6.52±0.06	6.71±0.06†	6.23±0.08	6.50±0.08‡				
Inorganic phosphorus, mg%	3.25±0.09	3.38±0.11‡	3.95±0.12	4.25±0.14‡				
Plasma Fe, μg%	163.1 ±3.31	268.6±6.4†	153.4 ±4.7	248.5 ±6.1†				
Haemoglobin, g%	16.67±0.28	23.15±0.18†	15.53±0.32	21.47±0.35†	12.9±0.38	14.25±0.22	13.14±0.42	17.28±0.40‡
Haematocrit, %	48. 3±0.82	66.7 ±0.26†	44.8 ±1.02	61.77±1.05†	35.3±0.98	39.44±0.69	35.7 ±1.16	46.20±1.15‡

*The resting values are determined on the blood taken 20 hours after the race. ± = standard error.
† = $p < 0.001$.
‡ = $p < 0.01$.

would not permit blood to be drawn before the main event; therefore, the samples were taken after 20 hours' rest on the following day. Blood samples were placed in suitable tubes and dried heparin was added as an anticoagulant. For glucose determination a mixture of sodium fluoride and ammonium oxalate was used to prevent glycolysis. (Ten grams of sodium fluoride + 2 g ammonium oxalate were dissolved and diluted in distilled water to 100 ml. Of this solution 0.2 ml was put into test tubes and dried.) The Hb was determined by a modified oxyhaemoglobin method (7), blood sugar by the Hagedorn–Jensen procedure (8), plasma iron content by the Ramsay method (9), plasma bilirubin by the Malloy–Evelyn method (10), and plasma proteins by the micro-Kjeldahl method and distillation (11). The Fiske–Subbarow method (12) was used to determine inorganic phosphate and the modified Kramer–Tisdall (13) method to determine serum calcium. For the determination of total solids about 5 g of plasma was introduced into a weighed platinum dish. The major portion of the water was expelled by heating the open dish on a water bath and heating was continued in an air oven at 100 to 105° C to constant weight. Plasma sodium and potassium levels were determined with the aid of a Zeiss "Flammenphotometer" and the Zeiss "Elko II" electrophotometer was used for all colorimetric readings.

Experimental

Experiment 1. The Effect of Muscular Exercise on Certain Plasma Electrolytes

From the data in Table I it is evident that the plasma potassium, inorganic phosphate, and the serum calcium levels were in every case significantly increased immediately following muscular exercise.

A significant increase in plasma iron content, haematocrit value, and haemoglobin concentration was observed after exercise. A pronounced increase occurred in Hct value and Hb concentration as a result of exercise, especially after racing. The greatest increase during training was 33.5% in Hct and 11.2 g% in Hb, and during racing, 29.7% in Hct and 9.42% in Hb. The expected correlation between Hct and Hb concentration was well demonstrated; however, the correlation coefficient of the race horses after exercise, and especially in the samples taken immediately after racing was typically lower.

TABLE II

Correlation coefficient ("r" values) and standard error for hemoglobin concentration and haematocrit of horses in training and during racing

	Resting	After exercise
1. Regularly ridden horses	0.93 ± 0.036	0.95 ± 0.025
2. Race horses		
In training	0.96 ± 0.018	0.86 ± 0.055
In race	0.94 ± 0.013	0.79 ± 0.077

TABLE III

A comparison of haematocrit values, plasma solids, and plasma protein concentration after severe muscular excercise in human* and horse

	No. of cases	Plasma solids, g/liter		Plasma protein, g/liter		Haematocrit, %	
		Rest	Work	Rest	Work	Rest	Work
1. Experiences with human subjects	10	86.1	93.4	68.6	76.0	44.5	47.0
Δ			7.3		7.4		2.5
2. Present results with horses	8	88.0	89.2	60.1	64.7	35.7	46.2
Δ			1.2		4.6		10.5

*Data of Dill *et al.* (2)

TABLE IV

The effect of galloping on plasma bilirubin and blood glucose of horses in training and during racing

	Plasma bilirubin, mg%		Blood glucose, mg%	
	Resting*	After exercise	Resting*	After exercise
1. Regularly ridden horses				
Light gallop	1.32±0.36	1.36±0.32	90.4±1.30	103.0±1.44
Forced gallop			94.8±1.78	91.7±1.01
2. Race horses				
In training	1.42±0.14	1.44±0.14	103.3±1.59	164.2±9.90†
In race	3.13±0.16	1.55±0.13†	95.6±2.87	142.1±6.75†

*The resting values are determined on the blood taken 20 hours after the race. ± = Standard error.
† = $p < 0.001$.

The possibility must be considered that intensive muscular exercise of short duration caused pronounced sweating in several horses which could lead to a haemoconcentration and thus account for the observed increases in plasma elecrolytes. As we have already mentioned, Dill *et al.* suggested that during muscular exercise in human subjects the changes are referable to loss of water from the blood. Indeed, they predicted a change in red cell volume values from the change in serum solids and protein concentration. However, these results do not agree with the data presented here for horses.

Experiment 2. The Effect of Muscular Exercise on the Plasma Bilirubin and Blood Sugar Levels

Table IV indicates that the plasma bilirubin concentration during a 20-hour period following a race is approximately double that observed during the resting state. The changes in the blood sugar level following muscular exercise differed between the two groups of animals. In the case of race horses following training exercise or racing, the blood sugar level was significantly increased. In several cases it reached more than 200 mg%.

On the other hand, in the case of the regularly ridden horses a similar increase could not be detected. In horses exercised at an easy gallop an average increase of 12% was found in the blood sugar level, but this increase was not significant. When exercised at a forced gallop there was usually no increase, and in several cases there was a definite decrease. Red cell fragility tests were made on samples drawn immediately after the races and after 20 hours' recovery. Immediately after exercise the red cell fragility test value increased to 0.63 (haemolysis in 0.63% NaCl) from a resting value of 0.47.

On several occasions, it was observed that those horses that exhibited excitement while still confined in their boxes, before the training, had Hb and blood sugar concentrations which were almost identical with those of samples

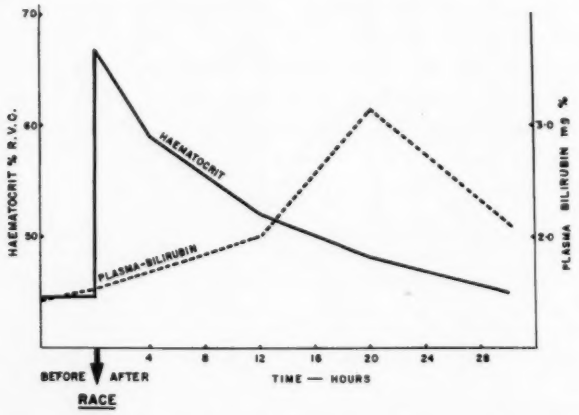


FIG. 1. The effect of exercise (racing gallop) and subsequent recovery on plasma bilirubin concentration and haematocrit.

TABLE V

The influence of exercise intensity on haematocrit, haemoglobin, and blood glucose values

Horses	Haematocrit, %			Haemoglobin, g%			Blood glucose, mg%		
	Rest in box	After a few steps	After training	Rest in box	After a few steps	After training	Rest in box	After a few steps	After training
1.	45.3	50.9	66.0	15.1	17.0	22.0	99	112	169
2.	44.3	44.4	63.2	15.0	15.1	20.2	106	99	258
3.	49.0	49.0	69.8	17.1	16.9	24.0	114	94	200
4.	45.1	52.8	69.8	15.4	18.5	23.2	109	159	176
5.	46.0	55.9	65.9	15.6	19.3	23.5	103	207	127

taken after the exercise. For example, the blood levels for one horse (No. 3) before training exercise were: Hct 66.2%, Hb 22.8 g%, and blood glucose 180 mg%. After a few days, the true resting blood samples showed the following levels: Hct 45.6%, Hb 15.6 g%, and blood sugar 102 mg%. Table V shows the blood levels in race horses at rest in the horse box, after saddling and cantering a few steps, and after training exercise.

These results indicate that there is a great variation among horses in their response to the preparation for training or racing. It is interesting to note that horse No. 3 on another occasion showed a considerable increase in Hb concentration and hyperglycaemia in the blood sample drawn in the box. Therefore it is suggested that the variations could occur also in the same horse at various physical states.

Discussion

Although no significant changes have been found in blood Hb content and Hct in experiments on human subjects after muscular exercise or racing, there is no doubt that in the case of race horses the changes occurring during racing in these blood components have some significance. Recently, Alexandrova (14) reported that during exercise of short duration the Hb concentration and the red cell count of horse blood were increased together with the white blood cells. During intensive work of long duration the Hb concentration showed hardly any increase; the red cell count increased, but the saturation was quite markedly decreased. In our experiments, the red cell count of the race horses at rest averaged about 13 millions/mm^3 and increased to an average of about 18 millions/mm^3 immediately after the races. A similar increase in the white cell count was observed in 19 horses after racing (WCB, 9500) but the count decreased slightly after 20 hours' rest (to 8200). The iron content of the centrifuged red cells was unchanged compared with the resting value. Evans *et al.* (15) reported that, in their experiments, the haematocrit value increased after an easy gallop to approximately 46.2% from a resting value of 32.5%. The increases in plasma adrenaline and noradrenaline were highly significant, but no increase in blood sugar levels was found.

The changes reported here in the plasma electrolyte concentration, in general, correlate with similar data found in the literature. Havard and Reay (16) first reported that immediately following exercise of short duration there is a rise in the blood phosphate level followed at once by a fall to below the resting level. This fall was less in trained than in untrained individuals. Increased inorganic phosphorus and serum calcium levels were also reported by Owles (17) and Dill *et al.* (2) following muscular exercise. In these experiments we have found the plasma sodium, potassium, inorganic phosphorus, and serum calcium levels increased during exercise or racing.

The observed increase in electrolytes cannot be explained as a result of stimulation of the sympathetic nervous system. Several experiments have

been reported which show that after an injection of epinephrine the plasma inorganic phosphorus and potassium levels decrease. However, it has been demonstrated here that plasma potassium, inorganic phosphorus, and serum calcium increase quite significantly as a result of muscular exercise. The increase in plasma potassium level is well known and is related to muscular exercise per se. Fenn (18) reported that muscular contraction resulted in a release of potassium from muscle fibers. Farber (19) and Grob (20) reported similar findings in human subjects. In a recent paper (21) we have reported that the plasma potassium increase following muscular exercise in rats varies directly with the intensity of the exercise, and that the sodium concentration decreases. In the experiments on race horses reported here, the plasma sodium level showed only a slight average increase; however, this change was not significant. At present there is no explanation for this observation, but attention is called to Blake's report (22) on the effect of exercise on renal haemodynamics, water, and sodium excretion in dogs. He found that moderate exercise did not influence renal function, but that when exercise was shown to be related to an emotional component, a well-defined reduction in sodium excretion was observed. It seems probable that an "emotional stress" causes an increase in the rate of reabsorption of sodium, since decreased excretion did not correlate with similar changes in glomerular filtration rate.

The results of our experiments show that regularly ridden horses have a significantly lower resting Hb level than that observed with race horses, while the degree of increase resulting from exercise corresponds to that reported in the literature. Increased intensity of exercise results in increased haematocrit values. After severe exercise the Hb concentration achieved the same level as in race horses at rest. Contrary to the results reported for human subjects, especially athletes, it was found that the Hb concentration, even at rest, is very much dependent on the state of training. In previous experiments on farm horses (23) it was found that the resting values of Hb and Hct were 11.4 g% and 32.23% respectively. This compared with the resting values found here of 12.9 g% and 35.3% for regularly ridden horses, and 15.5 g% and 44.8% for race horses. It appears that the changes in the blood components produced by exercise in trained race horses and the changes in those of regularly ridden but not systematically trained horses are quite dissimilar. In the former group, at the very start of the race or even before the start, large numbers of red cells from the blood reservoir and bone marrow enter the blood stream thus increasing the Hb concentration.

These last results emphasize the idea that the presence of emotional factors is necessary to the increase in blood sugar level. This idea has been mentioned by Best and Partridge (24) in their experiments on atheletes performing in the Olympic games. The increased reacting capacity of the race horses to every external stimulation is well known. This is typified by "starting fever", a phenomenon presumably depending on conditioned reflexes to a

previously known image (i.e. saddling, bridling, catching sight of the jockey, etc.) which eventually result in mobilization of red blood cells and increased blood sugar level. This phenomenon does not occur before the start in well-trained and conditioned race horses, but it is probable that the same physiological results could occur after the horse has taken only a few steps. The increase in blood sugar level is not a primary requisite for muscular work, since no definite increase was observed in the regularly ridden horses. Edwards *et al.* (25) reported that hyperglycaemia is uncommon during exercise with little or no emotional stress. They observed a definite hyperglycaemia in football players when the game was half over. According to Bøje (26) the blood sugar level at average working speed remains at the resting level or slightly decreases, but increases with increasing intensity of work. Evans *et al.* (15), in their experiments with horses, found no increase in blood sugar level after muscular exercise. Nor did Black (27), in his experiments with fish, find significant changes in blood glucose following 15 minutes of vigorous exercise. In our experiments on rats performing similar exercise (28) the blood sugar level showed little increase over control animals.

In blood samples drawn from horses after the race, the decreased resistance of the red blood cells was quite notable. One explanation for this phenomenon might be that the organism mobilized old cells from the depot or reservoir. On the other hand, cells might be mobilized from the bone marrow, and these immature red cells, having an even smaller capacity for resistance, quickly disintegrated causing a transitory hyperbilirubinaemia. It was found, in the experiments reported here, that the red cells mobilized during exercise were quite different in several respects from those found in the peripheral blood stream at rest. In 1940, Ludány *et al.* (29) reported that blood from the spleen showed different physical, chemical, and serological characteristics from the peripheral blood. Jokl (30) presumed that the blood cells from the reservoir entering the blood stream as a result of muscular exercise differed from the rest of the red cells to a large extent according to their age. In the present work, a definite increase in Hct values was observed after a few minutes of forced gallop. Therefore, it appears that, in race horses, the red cell mobilization is pronounced and occurs at the beginning of the race. The previously reported (31) experiments on catalase activity in blood are of interest in connection with this work. In the race horses, it was found that the blood catalase activity increased as a result of muscular exercise. This increase is considered to be due to the catalase activity of the red cells mobilized during the racing performance. At rest, the average reaction rate K_{10} was 0.0565, but after the race the average value increased to 0.0792. This increase in average K_{10} value is not in direct relationship with the increased values found for Hb and Hct. This fact, together with the change in red cell fragility test values and the alteration in the relationship between Hb and Hct values following a training or race, tends to support the theory that, in race horses, the blood cells entering the circulation are qualitatively different from the peripheral blood cells.

Acknowledgments

The author is very grateful to Dr. Dezsö Fehér, Tamás Flandorfer, István Monostori, and Dr. Imre Ócsag for their assistance. The valuable contribution in technical assistance given by Mrs. Eve Szaszovszky is also gratefully acknowledged.

References

1. IZQUIERDO, J. J. and CANNON, W. B. Am. J. Physiol. **84**, 545 (1928).
2. DILL, D. B., TALBOTT, J. H., and EDWARDS, H. T. J. Physiol. **69**, 267 (1930).
3. STEINHAUS, A. H. Physiol. Revs. **13**, 103 (1933).
4. SCHEUNERT, A. and KRZYWANEK, F. W. Pflüger's Arch. ges. Physiol. **213**, 198 (1926).
5. IRVINE, C. H. G. J. Am. Vet. Med. Assoc. **133**, 97 (1958).
6. BRENON, H. C. J. Am. Vet. Med. Assoc. **133**, 102 (1958).
7. SRÉTER, F. Kisérletes Orvostudomány, **3**, 271 (1954).
8. HAGEDORN, H. C. and JENSEN, B. N. Biochem. Z. **135**, 46 (1923).
9. RAMSAY, W. N. M. Biochem. J. **53**, 227 (1953).
10. MALLOY, H. T. and Evelyn, K. A. J. Biol. Chem. **119**, 481 (1937).
11. HAWK, P. B., OSER, B. C., and SUMMERSON, W. H. Practical physiological chemistry. The Blakiston Co., New York. 1954.
12. FISKE, C. H. and SUBBAROW, Y. J. Biol. Chem. **66**, 375 (1925).
13. RAPPAPORT, F. Klin. Wochschr. **12**, 1774 (1933).
14. ALEXANDROVA, A. Konyevodstvo, Moscow, **11**, 30 (1952).
15. EVANS, L. C., SMITH, D. F. G., and WEIL-MALHERBE, H. J. Physiol. **132**, 542 (1956).
16. HAVARD, R. E. and REAY, G. A. J. Physiol. **61**, 35 (1926).
17. OWLES, W. H. J. Physiol. **69**, 214 (1930).
18. FENN, W. O. Physiol. Revs. **16**, 450 (1936).
19. FARBER, S. J., PELLEGRINO, E. D., CONAN, N. J., and EARLE, D. P. J. Med. Sci. **221**, 678 (1951).
20. GROB, D., LILJESTRAND, K., and JOHNS, R. J. Am. J. Med. **23**, 340 (1957).
21. SRÉTER, F. and FRIEDMAN, S. M. Can. J. Biochem. and Physiol. **36**, 333 (1958).
22. BLAKE, W. D. Am. J. Physiol. **165**, 149 (1951).
23. SRÉTER, F. and BARNA, J. Állatteny. Budapest, **3**, 271 (1954).
24. BEST, C. H. and PARTRIDGE, R. C. Proc. Roy. Soc. (London), B, **105**, 323 (1930).
25. EDWARDS, H. T., RICHARDS, T. K., and DILL, D. B. Am. J. Physiol. **98**, 352 (1931).
26. BØJE, O. Skand. Arch. Physiol., Suppl. **10**, 1 (1936).
27. BLACK, E. C. J. Fisheries Research Board Can. **14**, 117 (1957).
28. SRÉTER, F. and FRIEDMAN, S. M. Can. J. Biochem. and Physiol. **36**, 1193 (1958).
29. LUDNÁN, G., GORECZKY, L., and SÁRFY, E. Magyar Orvosi Archivum, Budapest, 5 (1940).
30. JOKL, E. Arbeitsphysiologie, **4**, 379 (1931).
31. SRÉTER, F. and SZÉKY, P. Reports of the Faculty for Animal Husbandry, Hungarian University of Agricultural Science (Budapest), **6**, 305 (1956).

THE POOL SIZE, TURNOVER RATE, AND OXIDATION RATE OF BODY GLUCOSE IN ANESTHETIZED WARM- AND COLD-ACCLIMATED RATS EXPOSED TO A WARM ENVIRONMENT[1]

FLORENT DEPOCAS
With the technical assistance of V. SECOURS

Abstract

The size and space of the body glucose pool along with its turnover and oxidation rates have been measured in anesthetized 30° and 6° C acclimated rats by a method involving continuous intravenous injection of small amounts of D-glucose uniformly labelled with C^{14} and attainment of relatively constant specific activities of plasma glucose and respiratory CO_2. Values of glucose pool space in warm-acclimated rats (essentially normal animals) were in accord with those found in the dog by a similar method. Results obtained on warm-acclimated rats indicated that previous published values of turnover and oxidation rates of glucose for normal rats were high by a factor of approximately 2 to 4. There was, however, close agreement between the values of turnover time of body glucose pool measured by the continuous infusion procedure and those obtained by others with the single intravenous or intraperitoneal injection procedure. In cold-acclimated rats, average absolute values of glucose pool size were significantly smaller than in warm-acclimated rats but the difference was lost when results were related to body weight. Small, non-significant differences in values of glucose pool size per 100 g body weight and in plasma glucose concentration combined to give a significantly larger glucose space in cold- than in warm-acclimated rats. Glucose turnover and oxidation rates, the ratio between these two quantities, and the proportion of respiratory CO_2 derived from glucose oxidation were not significantly different in the two groups of rats, thus indicating that cold acclimation is not associated with major alterations in glucose metabolism at least when studied on fully fed anesthetized animals at 30° C.

Introduction

Quantitative modifications of glucose metabolism are produced by prolonged exposure of white rats to cold environments. Baker and Sellers (1) found a decreased glycogen content of heart, liver, diaphragm, and perirenal fat but no change in the skeletal muscle glycogen and in the blood glucose level of rats after a 2-hour fast following exposure for 45 days to 1.5° C. Mansour and Hewitt (2) found hyperglycemia in rats fasted for 24 hours following exposure to about 5° C for 9 days. Increased sensitivity of cold-exposed rats to insulin has also been observed by both groups under the same conditions (1, 2). According to Pagé and Chénier (3) cold-acclimated rats (3 weeks at 8° to 10° C), after fasting for 26 hours at room temperature, gave a lower respiratory quotient at 29° C than similarly treated room temperature rats. Rats acclimated to cold and fed a high carbohydrate or high fat diet showed higher values of fasting liver glycogen, higher rates of intestinal absorption of glucose, and a greater deposition of glycogen in liver, at comparable rates of glucose absorption, than controls kept at room temperature

[1]Manuscript received September 23, 1958.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 5021.

(4, 5). Intraperitoneal or intravenous injection of D-glucose uniformly labelled with C^{14} (U-C^{14}-glucose) into non-fasted 6° and 30° C acclimated rats at −5° C resulted in a respiratory $C^{14}O_2$ pattern which was dependent on the acclimation temperature of the animals (6). Cold-acclimated rats exhaled less of the injected C^{14} as CO_2 in 2.5 hours under those conditions.

These diverse observations point to alterations in the eventual fate of dietary glucose induced by acclimation to cold environments, but direct measurements of the parameters of the more dynamic functions of glucose metabolism in warm- and cold-acclimated rats are needed for adequate elucidation of the fundamental metabolic effects of acclimation. In a previous study (7), an increase in the turnover of plasma glucose was found in anesthetized warm-acclimated rats transferred from a warm to a cold environment, but no significant change could be detected in similarly treated cold-acclimated rats. In this study, we have measured the pool size of body glucose along with its turnover and oxidation rates in anesthetized warm- and cold-acclimated rats maintained at 30° C, by a technique involving continuous infusion of U-C^{14}-glucose. Application of this method has heretofore been limited to the dog (8, 9) owing to the ease of obtaining serial samples of large volumes of blood and to the fact that this animal can be trained to calmly withstand the lengthy constant infusion procedure, thus obviating anesthesia. Previous demonstration of the retainment of certain features of acclimation to cold in barbital-anesthetized rats (10) and development of a simple chromatographic procedure for isolating small amounts of plasma glucose for estimation of specific activity (7) have made this study feasible in the anesthetized rat.

The present experiments were patterned after those of Searle *et al.* (8, 11) with the exception that the initial measurements of the turnover time of body glucose were made on different groups of rats than those used in the continuous infusion work. Essentially, the method first requires estimation of the average lifetime of a molecule of glucose in plasma (turnover time) from measurements of the rate of decrease of the specific activity of plasma glucose after a single intravenous injection of U-C^{14}-glucose. In the second step, the body glucose pool is initially labelled by injection of a priming amount of U-C^{14}-glucose followed by continuous injection of small amounts of labelled glucose at such a rate that the specific activity of plasma glucose is maintained at a constant value soon after start of infusion. Constancy of the specific activity of plasma glucose will be attained only when the ratio of activity in the priming dose to the activity injected per unit time is equal to the turnover time (11). Under these conditions the specific activity of the respiratory CO_2 is also expected to attain a constant value. These experiments then allow calculation of the size and space of the body glucose pool and calculation of its turnover and oxidation rates as demonstrated by Searle *et al.* (8, 11) and Steele *et al.* (9). In addition, the ratio of glucose oxidation rate to glucose turnover rate and the per cent respiratory CO_2 derived from glucose oxidation may be calculated. The exact meanings of the values measured by this method have been critically reviewed by Steele *et al.* (9).

Methods

Animals

Ten male Sprague–Dawley rats weighing on the average 216 g (range 189–252 g) were randomly assigned to two equal lots, one maintained at 30° C and the other at 6° C for an average of 65 days (range 51–78 days). The rats were kept in individual cages and Master Fox chow and tap water were supplied ad libitum. After acclimation, when the warm-acclimated rats weighed on the average 443 g (range 411–470 g) and the cold-acclimated rats 331 g (range 298–349 g), anesthesia was induced by intraperitoneal injection of sodium barbital at a dose of 290 mg per kg. Anesthesia was allowed to develop for 1 hour at 30° C (10). All experiments began at approximately 9.00 a.m. with animals in the fully fed state.

Experimental Design

The anesthetized rat was taped to a board in the supine position, the trachea was cannulated, and the right jugular vein exposed. After 20 minutes, a priming dose of U-C^{14}-glucose in saline was injected into the exposed right femoral vein through a 27-gauge hypodermic needle tip. The needle was connected to a polyethylene tube (Intramedic, PE20) leading from a 2-ml syringe mounted on a variable-speed infusion pump (Harvard Apparatus Co. Inc., model 600-900). The infusion pump was initially set to deliver 0.545 ml of the solution in 2.77 minutes for the 30° C rats and 0.493 ml in 2.49 minutes for the 6° C rats. Immediately following this initial injection, the pump was reset to deliver 0.00803 ml of the same solution per minute into the rats of the two acclimation groups. These ratios of priming dose to rate of continuous infusion had been obtained from estimates of average glucose turnover time calculated from specific activities of plasma glucose observed between the interval 60 to 120 minutes following a single intravenous injection of U-C^{14}-glucose into warm- and cold-acclimated rats at 30° C (7). The infusion pump was calibrated by colorimetric measurement at 450 mμ of a solution of recrystallized methyl orange in 0.01 *N* NaOH delivered under conditions identical with those used in the C^{14} work. Blood samples (0.25 ml) were obtained from the exposed jugular vein by direct puncture at intervals of approximately 30 minutes and transferred to tubes containing 0.02 ml of heparin solution (10 mg per ml). Specific activity of plasma glucose was obtained by methods described in detail in a preceding paper (7). Respiratory CO_2 was collected in 10% NaOH (6) during successive intervals of 30 minutes beginning 15 minutes after start of the constant infusion which was taken as time 0.

U-C^{14}-Glucose

U-C^{14}-glucose was obtained from Atomic Energy of Canada Ltd., Commercial Products Division, Ottawa. The substance was pure as determined by autoradiography of samples chromatographed on Whatman No. 1 filter paper with *n*-propanol – ethyl acetate – water (7:1:2) as solvent. The material had a specific activity of 67 μc per mg. Twenty-five to 50 μc of

C^{14} in 0.37 to 0.74 mg glucose were dissolved in 2.35 or 2.40 ml saline for injection into the animals during experiments lasting between 105 and 195 minutes.

Radioactivity Determinations

The C^{14} determinations on labelled glucose for injection and on plasma glucose isolated chromatographically were made by spotting aliquot portions of the solutions on aluminum planchets and drying in a current of air. Plates of dilutions of the initial injected solutions were of negligible solids content and thus showed true activity at infinite thinness. When aliquots of these solutions were diluted with water or plasma and carried through the usual chromatographic separation, the average recovery of C^{14} was $90.0 \pm 0.6\%$. Attempts to identify the nature of the loss by successive chromatography of the same U-C^{14}-glucose sample failed to give consistent estimates of the purity of the glucose or of the loss of glucose on chromatography. Counts on successive dilutions of the glucose eluted from the paper strips indicated that enough solids were eluted from the paper strips to lower the counting rate by a few per cent. These additive losses apparently summated to a constant 10% of the initial activity. Consequently, the counting rates of the plasma glucose samples, as measured with a Nuclear-Chicago Model D47 counter, were corrected for this over-all loss. At least 10,000 disintegrations were counted on each sample.

All barium carbonate samples from respiratory CO_2 were counted with a Nuclear-Chicago Model D34 Geiger counter. These counting rates were corrected to infinite thinness with correction factors calculated from measurements of specific activity of samples of barium carbonate, obtained by combustion of a diluted C^{14}-glucose sample of C^{14} content measured at infinite thinness before dilution with normal glucose.

The efficiency ratio of the two counters used in this work was calculated, for each series of samples from each rat, from counts on the radioglucose samples prepared for measurement of the C^{14} content of the injected solution. All counts were then referred to the D34 counter.

Results

Maintenance of Steady State

Figures 1 and 2 illustrate the values for amount of CO_2 respired, for concentration of plasma glucose, and for specific activities of respiratory CO_2 and plasma glucose observed in the 30° and 6° C acclimated rats. In order to assess attainment of steady state in specific activities of plasma glucose (from 35 to 180 minutes) and respiratory CO_2 (from 75 to 195 minutes) the linear coefficients of regression of these quantities on time after start of infusion of C^{14} were determined for individual rats by the method of least squares. Corresponding average regression coefficients of both sets of measurements for the 30° and 6° C rats did not differ significantly. Specific activity data for plasma glucose for all rats (except No. 372 which was not included in this analysis because of the paucity of data on this animal) gave

mutually compatible regression coefficients averaging 0.010 ± 0.008 specific activity unit (per cent C^{14} infused per minute found per mg glucose carbon) per hour. This average regression coefficient was not different from zero, thus indicating adequate maintenance of the steady state of specific activity of the plasma glucose.

Analyses of covariance also indicated that the regression coefficient for individual animals in respect to specific activity of respiratory CO_2 were not significantly different. Data for four 30° C rats (Nos. 382, 530, 474, and 495) and three 6° C rats (Nos. 370, 540, and 597) gave a weighed average regression coefficient of 0.008 ± 0.002 specific activity unit (per cent of the injected C^{14} per minute found per mg respiratory CO_2 carbon) per hour which was significantly different from zero.

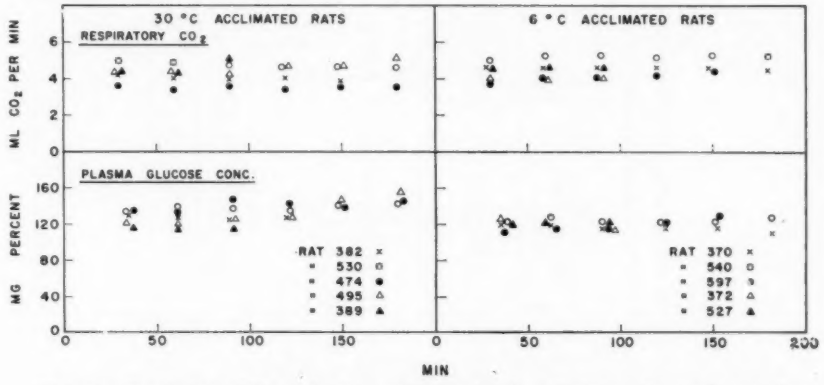


FIG. 1. Respiratory CO_2 production and concentration of plasma glucose in anesthetized 30° C and 6° C acclimated rats during continuous infusion of glucose uniformly labelled with C^{14} at 30° C.

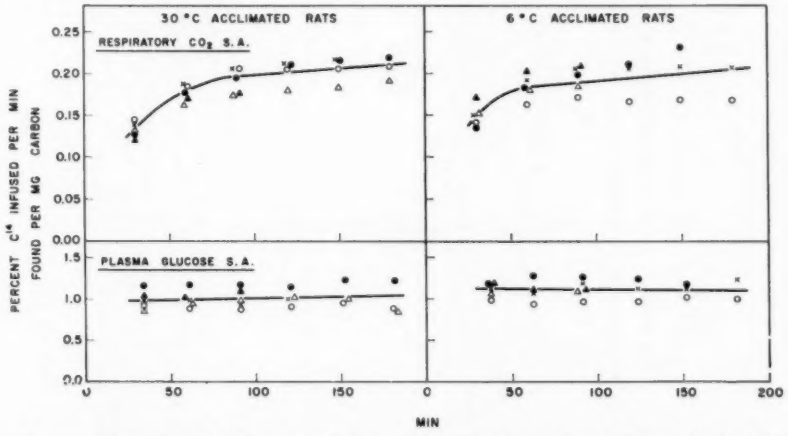

30 °C ACCLIMATED RATS
6 °C ACCLIMATED RATS
RESPIRATORY CO2 S.A.
PLASMA GLUCOSE S.A.
PERCENT C14 INFUSED PER MIN FOUND PER MG CARBON
MIN

FIG. 2. Specific activities of respiratory CO_2 and plasma glucose in anesthetized 30° and 6° C acclimated rats during continuous infusion of glucose uniformly labelled with C^{14} at 30° C. Symbols are as in Fig. 1.

TABLE I

Average concentration of plasma glucose, glucose pool size and space in anesthetized 30° and 6° C acclimated rats

	Rat No.	Time of infusion, min	Weight, g	Av. plasma glucose concn., mg%	Glucose pool size		Glucose space, % b. wt.
					mg C	mg C/100 g b. wt.	
30° C	382	165	411	126	64.6	15.7	31.1
	530	195	470	138	69.5	14.8	26.9
	474	195	407	141	53.2	13.1	23.2
	495	195	460	134	66.8	14.5	27.1
	389	105	468	116	59.7	12.8	27.3
	Mean ± s.d.		443.2 ± 12.0	131.0 ± 3.4	62.8 ± 2.4	14.2 ± 0.6	27.1 ± 1.0
6° C	370	195	340	115	51.0	15.0	32.5
	540	195	344	125	58.5	17.0	34.1
	597	165	323	119	46.9	14.4	30.4
	372	105	349	120	52.9	15.2	31.6
	527	105	298	122	50.1	16.7	34.4
	Mean ± s.d.		330.8 ± 12.0	120.2 ± 3.4	51.9 ± 2.4	15.7 ± 0.6	32.6 ± 1.0
30° C–6° C			112.4 ± 16.9*	10.8 ± 4.9	10.9 ± 3.4†	1.5 ± 0.8	−5.5 ± 1.5*

*Difference significant at the 1% level.
†Difference significant at the 5% level.

TABLE II

Turnover and oxidation rates of plasma glucose in anesthetized 30° and 6° C acclimated rats

		Turnover rate		Oxidation rate			
	Rat No.	mg C/min	mg C per min per b. wt.$^{0.6}$ $\times 10^2$	mg C/min	mg C per min per b. wt.$^{0.6}$ $\times 10^2$	Turnover rate $\times$ 100 / Oxidation rate	% respiratory CO_2 derived from glucose oxidation
30° C	382	0.95	2.57	0.42	1.15	44.5	20.1
	530	1.03	2.56	0.53	1.32	51.7	21.1
	474	0.79	2.13	0.31	0.86	40.4	16.8
	495	0.98	2.48	0.44	1.13	45.5	17.1
	389	0.88	2.20	0.45	1.12	51.0	15.6
	Mean ± s.d.	0.92 ± 0.04	2.39 ± 0.13	0.43 ± 0.03	1.12 ± 0.06	46.6 ± 1.7	18.1 ± 1.0
6° C	370	0.83	2.52	0.41	1.24	49.5	16.9
	540	0.95	2.87	0.45	1.36	47.5	15.9
	597	0.76	2.38	0.36	1.14	47.9	16.2
	372	0.86	2.56	0.39	1.06	45.4	15.7
	527	0.81	1.94	0.36	1.06	43.9	16.9
	Mean ± s.d.	0.84 ± 0.04	2.45 ± 0.13	0.39 ± 0.03	1.17 ± 0.06	46.8 ± 1.7	16.3 ± 0.3
30° C–6° C		0.08 ± 0.06	−0.06 ± 0.18	0.04 ± 0.04	−0.05 ± 0.09	−0.2 ± 2.3	1.8 ± 1.1

Glucose Pool Size and Space

Table I lists the individual values of average plasma glucose concentration, glucose pool size, and glucose space obtained for each rat in both acclimation groups. The average concentrations of plasma glucose were not significantly different in the two acclimation groups. There was a significantly larger absolute glucose pool in the 30° C rats, but this difference was lost when the pool sizes were calculated per 100 g body weight. Non-significant differences in concentration of plasma glucose and in pool size per 100 g body weight combined to give a significantly larger glucose space in cold-acclimated rats.

Turnover and Oxidation Rates of Body Glucose

Table II lists the calculated values of glucose turnover rate, oxidation rate, ratio of oxidation rate to turnover rate, and per cent respiratory CO_2 derived from glucose oxidation for individual rats. No significant differences were found between the average values of any of these quantities in each acclimation group, whether the raw data or results calculated per body weight to the 0.6 power (9) were compared.

Discussion

On the average the specific activity of plasma glucose was maintained at a constant value in warm- and cold-acclimated rats when the experimental procedure of Searle *et al.* (8) was used. Corresponding specific activities of respiratory CO_2, however, were correlated significantly to time of observation. The average regression coefficient for the two groups of rats (0.008 ± 0.002 specific activity unit per hour) indicated a slow increase in specific activity of approximately 4% per hour on the linear part of the curves (Fig. 2). This was undoubtedly a reflection of an increase in labelling of substrates eventually oxidized to CO_2 but not by pathways directly involving glucose. The glucose oxidation rate was thus in error by approximately the same amount when the average specific activity of respiratory CO_2 observed between 75 and 195 minutes after beginning of infusion was used in the calculations. No attempt was made to correct the rates of oxidation of glucose for this effect.

Values of glucose pool size for normal non-fasted rats, obtained from measurements of specific activities of blood glucose following a single intravenous (12) or intraperitoneal (13) injection of U-C^{14}-glucose have been given respectively as 127 and 66 mg glucose per 100 g body weight (Table III). These values are much higher than the average of 35.5 ± 1.5 mg per 100 g body weight found for warm-acclimated rats in this study. This is in accord with results obtained by Searle *et al.* (11) when applying both techniques (single injection and continuous infusion) to the dog. Errors involved when glucose pool size and volume of distribution of glucose are calculated from the estimated value at 0 time of the specific activity of glucose in the single-injection method have been discussed at length by Searle *et al.* (11).

The glucose space found in anesthetized warm-acclimated white rats ($27.1 \pm 1.0\%$ of body weight) agrees well with values obtained by using the same technique with the dog (9, 11). This is in accord with the concept

TABLE III

Values of parameters of glucose metabolism in non-fasted white rats as determined by single-injection and continuous-infusion techniques

Method	Ref.	Pool size, mg glucose/ 100 g b. wt.	Turnover rate, mg glucose per hr per 100 g b. wt.	Turnover time, hours	Oxidation rate, mg glucose per hr per 100 g b. wt.	% respiratory CO_2 derived from glucose oxidation
Single intravenous injection	5	127	103	1.23	69	45
Single intraperitoneal injection	11	66	59±12	1.12	—	—
Continuous infusion	Present work*	35.5±1.5	31.3±1.3	1.13	14.9±0.9	18.1±1.0

*Values found in anesthetized warm-acclimated rats.

that the glucose pool is distributed mainly in the extracellular fluid, as the sodium space found previously in white rats after 5 weeks' acclimation to 30° C was 23.4% of body weight (14).

The turnover rate of plasma glucose, which is obtained from the ratio of glucose pool size to the turnover time in the single-injection method or, alternatively, from the ratio of the counts infused per minute to the specific activity of plasma glucose measured in the continuous infusion method, is seen to vary considerably with the technique used (Table III). That the variation is almost entirely due to errors involved in estimating glucose pool size by the single-injection method is indicated by the close agreement between the turnover times measured with the single-injection method after intravenous or intraperitoneal injection of the labelled glucose and by the continuous-infusion method in non-fasted normal or anesthetized rats (Table III).

Comparison of rates of oxidation of glucose obtained by the single-injection method and the continuous-infusion method applied to normal dogs by Searle *et al.* (8) indicated that the single-injection method gave results high by a factor of 2.5 to 3. The ratio between the rates of oxidation obtained by the two methods with the rat (Feller *et al.* (12) and present work) is 4.6.

The present work does not indicate major differences in the metabolism of glucose in anesthetized, fully fed, warm- and cold-acclimated rats at 30° C. The average absolute glucose pool size was significantly smaller in the cold-acclimated rats, but the pool size expressed per 100 g body weight was slightly larger on the average in the 6° C rats, although the difference was not significant. This observation adds weight to the proposition of Steele *et al.* (9) that pool size must be related to body weight. The non-significant differences between average glucose pool size per 100 g body weight in each group of rats and also between average concentrations of plasma glucose combined to give a significantly larger glucose space in the 6° C acclimated rats. This is qualitatively in accord with the results of Deb and Hart (14) which indicate a significantly larger extracellular fluid volume, relative to body weight, in cold-acclimated rats (26.0 ± 0.7 ml per 100 g) than in warm-acclimated rats (23.6 ± 0.7 ml per 100 g) after an exposure of 5 weeks.

Some of the differences in carbohydrate utilization found by previous workers (2, 3, 4, 5) in warm- and cold-acclimated rats were obtained with the fasted animals. A study of the above parameters of glucose metabolism in fasted warm- and cold-acclimated rats might, therefore, be profitable. Also, it would be interesting to investigate possible changes in the rates of turnover and oxidation of body glucose associated with different exposure temperatures in the unanesthetized rat. Such studies are now in progress in this laboratory.

Acknowledgment

Statistical analysis provided by the Biometrics Section of this Division is gratefully acknowledged.

References

1. BAKER, D. G. and SELLERS, E. A. Am. J. Physiol. **174**, 459 (1953).
2. MANSOUR, T. E. and HEWITT, W. F., JR. Endocrinology, **54**, 20 (1954).
3. PAGÉ, E. and CHÉNIER, L. P. Rev. can. biol. **12**, 531 (1953).
4. PAGÉ, E. and BABINEAU, L. M. Can. J. Biochem. and Physiol. **32**, 395 (1954).
5. PAGÉ, E., BABINEAU, L. M., and LACHANCE, J. P. Rev. can. biol. **14**, 144 (1955).
6. DEPOCAS, F., MACLEOD, G. K., and HART, J. S. Rev. can. biol. **16**, 83 (1957).
7. DEPOCAS, F. Can. J. Biochem. and Physiol. **37**, 175 (1959).
8. SEARLE, G. L., STRISOWER, E. H., and CHAIKOFF, I. L. Am. J. Physiol. **185**, 589 (1956).
9. STEELE, R., WALL, J. S., DEBODO, R. C., and ALTSZULER, N. Am. J. Physiol. **187**, 15 (1956).
10. HÉROUX, O., HART, J. S., and DEPOCAS, F. J. Appl. Physiol. **9**, 399 (1956).
11. SEARLE, G. L., STRISOWER, E. H., and CHAIKOFF, I. L. Am. J. Physiol. **176**, 190 (1954)
12. FELLER, D. D., STRISOWER, E. H., and CHAIKOFF, I. L. J. Biol. Chem. **187**, 571 (1950).
13. RUSSELL, J. A. Perspectives in Biochemistry and Medicine, **1**, 138 (1958).
14. DEB, C. and HART, J. S. Can. J. Biochem. and Physiol. **34**, 959 (1956).

THE DESTRUCTION OF O,O-DIETHYL-S-2-DIETHYLAMINO-ETHYL PHOSPHOROTHIOLATE BY LIVER MICROSOMES[1]

J. F. SCAIFE AND D. H. CAMPBELL

Abstract

Liver homogenates prepared from the rat, rabbit, mouse, and guinea pig possess an enzyme system capable of destroying O,O-diethyl-S-2-diethylamino-ethyl phosphorothiolate at the rate of 150 to 200 μg/hr/g of fresh tissue. The homogenates prepared from the pig, dog, cow, and frog destroyed this compound at a rate of 50 to 100 μg/hr/g, but those prepared from man and the cat possessed negligible activity. Rat plasma, brain, kidney, diaphragm, whole gut, and spleen also possessed little or no activity. This enzyme system is located in the microsomes, disruption of which is accompanied by loss of enzymatic activity. The activity is dependent upon oxygen, inorganic phosphate, and diphosphopyridine nucleotide. Inhibitor studies indicate that enzymic SH-groups are necessary. The enzyme has no action on diisopropyl phosphorofluoridate, isosystox, or tetraethyl pyrophosphate, although these compounds are rapidly destroyed by the liver.

Introduction

The existence in various tissues of enzymes capable of hydrolyzing a number of specific anticholinesterases has been known for some time (1–6). The development of the anticholinesterase alkyl phosphorothiolates as insecticides, typified by O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate (DSDP) (7), raised the question of the possibility of their enzymatic destruction in the mammalian body.

This report is concerned with work demonstrating that DSDP is destroyed by an oxidative mechanism involving liver microsomes. It is further demonstrated that DSDP is not attacked by the enzymes which destroy the common anticholinesterases like diisopropyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP), and isopropyl methyl phosphorofluoridate (sarin). The nature of the enzyme, and its cofactor requirements, as well as its distribution among various species, including man, have been investigated.

Materials and Methods

Compounds

Sarin, DFP, TEPP, and DSDP were samples obtained from the Defence Research Chemical Laboratories, Ottawa. O,O-Diethyl-S-2-ethylmercaptoethyl phosphorothiolate (isosystox) was obtained from Suffield Experimental Station, Defence Research Board, Ralston, Alberta. DSDP was carefully purified from traces of pyrophosphates by washing with acidic and alkaline solutions and redistilling.

Beef and pork livers were taken from slaughterhouse animals during routine carcass preparation, after slaughter in the usual manner. They were immediately placed on ice and used within 1 hour, or else frozen. Samples

[1]Manuscript received August 11, 1958.
Contribution from the Defence Research Kingston Laboratory, D.R.B., Kingston, Ontario.
Issued as Report No. 61A.

of human liver were obtained at autopsy 1 or 2 hours after death. They were rapidly frozen in dry ice before being transported to the laboratory. Frogs, guinea pigs, rabbits, and rats were decapitated and the livers were removed as soon after death as possible. Dogs and cats were anesthetized with Nembutal prior to removal of the liver. Fresh livers were immediately perfused with ice-cold isotonic (0.25 *M*) sucrose solution, and homogenized in sucrose–phosphate buffer, containing nicotinamide. The frozen liver samples were not perfused. Homogenization was accomplished with a Potter–Elvehjem homogenizer fitted with a teflon pestle. Cellular debris was removed by centrifuging at 600 × *g* for 10 minutes.

The medium had the following composition, in grams per liter: potassium dihydrogen phosphate, 5.98; nicotinamide, 3.42; sucrose, 43.1. Sufficient potassium hydroxide was added to bring the pH to 7.3. The volume of medium used was equal to 2.5 times the weight of the liver sample.

Incubation of homogenates was carried out in a Dubnoff metabolic shaker (Precision Scientific, Chicago) or in a Warburg apparatus. The incubation mixture consisted of 1.5 ml of homogenate, 3.8 mg of diphosphopyridine nucleotide (DPN) in 0.1 ml, and cholinesterase inhibitor in 0.1 or 0.2 ml of water. The total volume was adjusted to 2.4 ml with buffer, the pH of which was 7.3, and incubated at 37° C, with shaking in a gas phase of 100% oxygen.

Reactions under anaerobic conditions were allowed to proceed in double-arm Warburg vessels which were maintained free from oxygen by flushing with purified oxygen-free nitrogen. At the end of the incubation period the reaction was stopped by the addition of 0.2 ml of an aqueous solution containing 5 g trichloracetic acid and 10 g sodium tungstate in 100 ml of water. This solution was not added to the vessels incubated under aerobic conditions. At the end of the incubation period all vessels were immediately placed in crushed ice and aliquots removed and diluted with water. In these samples the trichloracetic acid and the sodium tungstate were considerably diluted and control experiments revealed that these compounds then had no effect upon cholinesterase activity. Residual anticholinesterase activity of the diluted samples was determined manometrically as described below.

Cholinesterase activity was estimated manometrically under the following conditions; 1.5 ml of 0.05 *M* $NaHCO_3$, 0.5 ml of 0.6 *M* NaCl, 0.5 ml of enzyme (10% lysed human red cells), 0.1 ml of 0.3 *M* acetylcholine bromide, and 0.1 to 0.2 ml of inhibitor solution. The total volume was adjusted to 3.0 ml with water. The gas phase was 5% CO_2 in N_2, giving a pH of 7.43. The mixture of enzyme and inhibitor was preincubated for 15 minutes before the addition of substrate.

Unless otherwise stated, the word homogenate will be taken to refer to a homogenate prepared from the liver of a male albino rat weighing approximately 125 g. It is worth emphasizing that due to animal variability the enzymatic activity of individual homogenates is not a constant and reproducible value but is unique for each homogenate preparation. Consequently

the designation of absolute values for many of the measurements undertaken in this work would be meaningless, and instead effects have often been described in more general terms as they were evaluated from repeated comparative experiments with different homogenates.

Results

Table I shows the destruction of DSDP by a liver homogenate as measured by the loss of anticholinesterase activity. It will be seen that activity of the homogenate is dependent upon oxygen, no destruction occurring under anaerobic conditions. Destruction does not occur when the homogenate has been heated. The activity of individual homogenates is variable from preparation to preparation but destruction of DSDP is of the order of 200 μg/hr/g wet weight of rat liver.

TABLE I

Destruction of DSDP by rat liver homogenates

	μg/hr/g liver
Whole homogenate (O_2)	195
Whole homogenate (N_2)	5
Boiled homogenate	3

In order to be quite sure that destruction was not an artifact produced by homogenization an isolated intact rat liver was slowly perfused with a solution containing DSDP in physiological saline. The perfusing solutions were aerated with oxygen and maintained at 37° C for 2 hours. The whole liver and perfusing solution was then homogenized and an aliquot was tested against red cell cholinesterase (ChE) for residual DSDP. Comparison with DSDP solutions kept under identical conditions revealed that appreciable destruction had occurred under these conditions.

Since the homogenate is a heterogeneous system an attempt was made to localize and associate the activity with the subcellular fractions present in the homogenate. Separation was effected by means of the ultracentrifuge (8) as follows. The cellular debris was removed from the crude homogenate by centrifuging for 10 minutes at 600 $\times$ *g*. The homogenates used throughout this work were obtained after decantation. Further fractionation was effected by centrifuging for 15 minutes at 15,000 $\times$ *g* to sediment the mitochondria. A further centrifuging of the decanted layer for 45 minutes at 140,000 $\times$ *g* produced a pellet of microsomes. No activity was exhibited by any of these fractions alone. If the particulate phases were recombined with the soluble supernatant fraction activity was regained. However, if the mitochondria were resuspended in the medium and then resedimented several times they lost a large portion of their activity. There is, thus, reason to suggest that the activity of the mitochondrial fraction is due to microsomal contamination. The microsomal fraction has been shown (9, see also (10)), to be heterogeneous by electron microscope photographs and to consist of

granules of ribonucleic acid (RNA) spread along fragments of the endoplasmic reticulum. When a microsomal suspension is treated with sodium desoxycholate (9) the reticulum is solubilized together with many of its enzymes and the RNA particles freed. As is shown in Table II treatment of a homogenate

TABLE II

Destruction of DSDP by microsomes and modified microsome suspensions

System		μg/hr/g liver
Whole homogenate		216
Microsomes in supernatant		202
Microsomes in buffer		147
Desoxycholate-treated homogenate	supernatant	9
	RNA particles	28
Water extract of microsomes		13

with 0.5% sodium desoxycholate results in almost complete loss of activity. Attempts to solubilize the enzyme system by water extraction (11) were unsuccessful. Sonic vibration of the homogenate at a frequency of 10 kc, also caused a loss of enzymatic activity.

Conditions Necessary for Activity

As demonstrated in Table I oxygen is necessary for activity. Attempts to replace oxygen with methylene blue or H_2O_2 were unsuccessful.

In order to examine the conditions necessary for enzymatic activity, microsomes were separated from homogenates by differential ultracentrifugation. Sedimented microsomes were then suspended by gentle homogenization in buffer solution (9) or in supernatant treated in various ways. Microsomes were also separated by ultracentrifugation from homogenates prepared using a medium of isotonic (0.25 *M*) sucrose instead of the phosphate–sucrose–nicotinamide medium. With the proper addition of components necessary for activity these microsomes did not seem to differ in activity from those prepared in the regular buffer medium. Homogenates prepared in water or 0.9% saline were inactive.

Figure 1 shows the results obtained when microsomes were resuspended in supernatant which had been subjected to various treatments, or in buffers modified by the addition or deletion of certain components. The recombination has been designated active or inactive depending on whether or not it produces a destruction of DSDP of at least 50% of that obtained with a suspension of microsomes in supernatant. Two main points are evident. First, the recombination of microsomes with either dialyzed or boiled supernatant is still active, while the combination with boiled supernatant which had subsequently been dialyzed is inactive. Second, microsomes separated from a sucrose medium which also contains nicotinamide require only the addition of phosphate to allow enzymatic activity to proceed, while microsomes prepared from a sucrose medium not containing nicotinamide require the addition of both phosphate and DPN for activity.

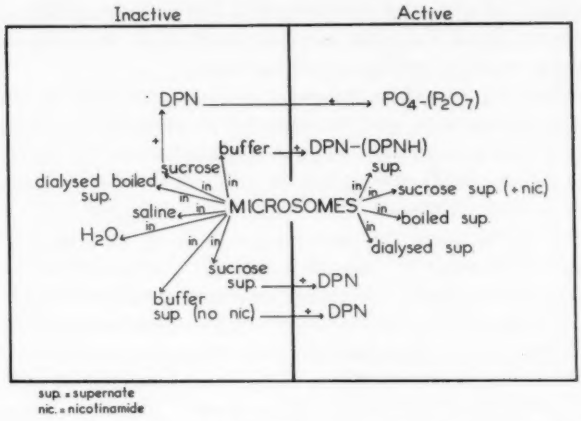


FIG. 1. Conditions under which liver microsomes are active or inactive with respect to the destruction of DSDP.

The conclusions to be made from this work are that both DPN and phosphate are necessary for activity. These deductions are supported by the fact that an active system can be prepared from washed microsomes resuspended in buffer to which DPN has also been added.

The substitution of pyrophosphate for orthophosphate does not affect the activity, nor does the replacement of K^+ by Na^+ in the medium. The addition of either magnesium or calcium is without effect upon the activity. The substitution of triphosphopyridine nucleotide (TPN) or its reduced form (TPNH) for DPN does not give systems with appreciable activity, but reduced diphosphopyridine nucleotide (DPNH) has been found to be equivalent to DPN. Increasing the concentration of DPN from 3.8 mg to 7.6 mg per reaction vessel produces approximately a 50% reduction in the activity of the homogenate. ATP can not replace DPN in this system.

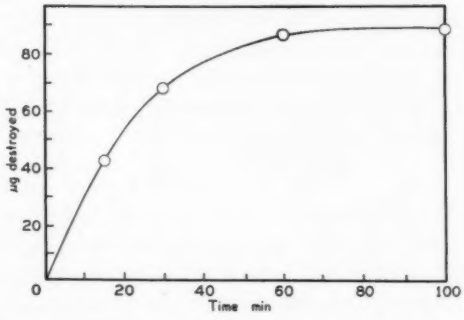


FIG. 2. Rate of destruction of DSDP by rat liver microsomes (1.5 ml of microsome suspension in buffer–supernatant).

Figure 2 shows the course of destruction of DSDP by microsomes suspended in supernatant. Similar curves are obtained with microsomes in buffer medium but the activity is then somewhat lower.

When determining the pH for optimum activity, microsomes were separated from a sucrose homogenate and resuspended in phosphate buffers (0.044 *M*) varying in pH from 5.1 to 8.3. There is little alteration in activity over the physiological range of pH values but it falls off sharply below pH 5.5 and above 8.3, with a maximum activity at approximately pH 7.7.

In order to characterize the microsomal enzyme the effect of numerous inhibitors upon the activity has been tested. Initial experiments carried out with microsomes suspended in the supernatant, prepared from homogenates made in the medium, indicated that interfering compounds in the supernatant altered the effect. This was particularly noticeable with the sulphydryl-reacting inhibitors and presumably is due to the presence of SH-compounds like cysteine or glutathione in the supernatant. Thus in the presence of supernatant, *p*-chlormercuribenzoate showed no inhibition of enzymatic activity while in the absence of supernatant it was a powerful inhibitor. Consequently the testing of all compounds for inhibitory effects was done in the absence of supernatant and the results are given in Table III. The percentage inhibition is that obtained from a 60-minute incubation period of microsome suspension and DSDP. The most effective inhibitors discovered in this investigation were the heavy metal ions (Cu^{++}, Hg^{++}, Ag^{+}), phenylhydrazine, sodium iodoacetate, sodium azide, SKF-525A, methylene blue, and cytochrome *c*.

The addition of coenzyme A (CoA), ascorbic acid, oxalacetate or acetate, singly or in combination, had no effect upon the activity. The addition of H_2O_2 or catalase did not affect the activity.

TABLE III

Effect of inhibitors on DSDP destruction

Inhibitor concentration: 1 mg in 2.4 ml total volume (unless indicated)

Per cent inhibition					
Low		Medium		High	
KCN	0	Alloxan	49	Cu^{++} (0.05 mg)	90
Na_3AsO_4	13	Versene	36	Hg^{++} (0.1 mg)	70
Na_3AsO_3	14	Adrenaline	52	Ag^{+} (0.1 mg)	90
2,4-Dinitrophenol	25	α,α'-Dipyridyl	52	Phenylhydrazine	95
n-Octyl alcohol	0	Merthiolate	57	*p*-Cl-Hg-benzoate (0.5 mg)	70
Dimedone	0			8-OH-quinoline	72
Hydroxylamine	0			Benzidine	86
Semicarbazide	15			Sodium iodoacetate	90
Phlorizin	7			NaN_3	98
				SKF-525A	99
				Methylene blue	89
				Cytochrome *c*	95
				N-Ethyl maleimide	76

Substrate Specificity

Liver homogenates contain high concentrations of enzymes of the DFP-ase type as well as the enzyme responsible for the destruction of DSDP, which make it difficult to determine whether this latter enzyme has any activity on the common anticholinesterases such as DFP or TEPP. A large amount of the hydrolytic activity of the homogenates can be eliminated by discarding the supernatant (1). Experiments were therefore conducted with a supernatant-free suspension of microsomes in buffer solution as previously outlined. The microsome suspension was incubated with DPN and the cholinesterase inhibitor in Warburg vessels. Duplicate series were run, one having an atmosphere of oxygen during the incubation and the other, an atmosphere of oxygen-free nitrogen. An increased destruction of the cholinesterase inhibitor in the presence of oxygen was taken to signify an activity of the DSDP-destroying enzyme on that inhibitor. Table IV shows the rate

TABLE IV

Destruction of isosystox, DFP, and TEPP by rat liver microsomes in buffer

	μg/hr/g liver	
	O_2	N_2
Isosystox	267	259
DFP	400	395
TEPP	195	193

of destruction of DFP, isosystox, and TEPP by a microsome suspension both in the presence and absence of oxygen. It will be seen that no increased destruction was found in the presence of oxygen. Since the microsomes still retain a high level of "sarinase", the enzyme responsible for the destruction of sarin, the rate of destruction of sarin was so great that any differential effect which might have been observed was lost. Thus it is not possible to draw any conclusion as to the activity of DSDP-destroying enzyme towards sarin.

No specific inhibitor could be found for the hydrolytic enzymes which did not also affect the activity of the preparation towards DSDP. In this connection it was found that the presence of KCN (1 to 2 mg per vessel) caused a marked acceleration in the rate of decomposition of DFP by microsomes, an action contrary to its effect with pure DFP-ase (12).

Enzyme Distributions in the Tissues

Rat tissues other than the liver have been examined for the presence of appreciable amounts of enzymatic activity towards DSDP. Tissues tested were brain, kidney, diaphragm, whole gut, and spleen. There was little or no activity in any of these organs or in plasma or gut contents. Likewise no activity was found in human plasma. In the rat, age or sex appeared to exert no influence on the ability of liver homogenates to destroy DSDP.

Enzyme Distribution among Animal Species

Using the liver homogenate technique activities of the order of 150–200 μg/hr/g liver have been found in the following species: rat, rabbit, mouse, and guinea pig. Lower levels of activity (50–100 μg/hr/g liver) were found in the pig, dog, and cow and slightly lower in the frog. However, a negligible activity was found in the cat and all six samples of human liver tested.

Discussion

The results clearly demonstrate that there exists in a number of animal species an enzyme or enzyme system capable of catalyzing the destruction of O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate by oxygen. This enzyme is not a hydrolytic enzyme like DFP-ase or sarinase, which can catalyze the hydrolysis of their substrates in the complete absence of oxygen. In fact the enzyme has no action on DFP, isosystox, or TEPP. In the rat it is located principally in the liver and occurs in high concentration in the livers of numerous other animal species. However, in some species, including man, this enzyme appears to be absent, or at least to exist in only negligible quantity. Other tissues from these species have as yet not been examined. It is worth noting that the samples of human liver were never obtained earlier than 1 hour post mortem and they were then frozen with dry ice for transportation. Experiments with rat and pig liver indicated that freezing had little effect upon activity. Moreover, rat livers left *in situ* for 2 hours after death and maintained at 37° C still showed a high degree of enzymatic activity. Consequently the lack of activity in human liver samples is considered to be a real effect and comparable with the lower activity exhibited by several other species.

The enzyme is located in the microsomal fraction of the liver homogenate. However, attempts to solubilize it were unsuccessful and suggest that this is an enzymatic function dependent upon particulate structural architecture.

The cofactor requirements indicate that either ortho-, or pyro-phosphate is necessary together with either DPN or DPNH. Normal liver homogenates contain phosphate but the experiments with washed microsomes prepared in isotonic sucrose clearly indicate that phosphate is necessary for activity. In the absence of nicotinamide, endogenous DPN is rapidly destroyed by the DPN-ase of liver (13). Hence activity is regained only by the addition of DPN to such nicotinamide-free homogenates. Evidently the DPN is bound in some non-dialyzable form in normal liver supernatant (14) and becomes dialyzable only after boiling.

The powerful inhibition produced by cytochrome *c* and methylene blue strongly suggests an interference in an electron transport system so that it may well be that the actual cofactor is DPNH and not DPN.

The effects of a variety of inhibitors on the enzyme clearly indicate that a SH-function is affected. This is also probably the explanation for the powerful effect of the heavy metal ions. Apparently a carbonyl function is not involved. The lack of inhibition by KCN indicates that the cytochrome

c – cytochrome oxidase system is inoperative, and that heavy metals like copper are not necessary for the activity. The inhibition produced by ethylenediamine tetracetic acid, disodium salt (versene) is in this case probably related to its solubilizing effect on the microsomes (9).

The tricarboxylic acid cycle would appear not to be involved and neither is active phosphorylation essential, as is suggested by the lack of activity shown by ATP.

The requirement for DPN and the lack of inhibition by *n*-octyl alcohol show that the enzyme is not a typical amine oxidase.

The inhibition produced by the compound SKF-525A (2-diethylaminoethyl diphenyl propyl acetate) is interesting in view of its inhibiting action on dealkylation mechanisms (15) and its terminal similarity to DSDP.

With the importance of this class of compounds as anticholinesterase agents for use as insecticides, the existence or lack of enzymatic activity towards them is clearly a factor which must be taken into account when considering their physiological action.

Acknowledgments

The author wishes to thank Dr. R. M. Heggie of Defence Research Chemical Laboratories, Ottawa, for the preparation of the agents used in this work, and Dr. P. A. Adie of Suffield Experimental Station for the sample of isosystox.

References

1. ADIE, P. A. Can. J. Biochem. and Physiol. **36**, 21 (1958).
2. AUGUSTINSSON, K. B. Biochim. et Biophys. Acta, **13**, 303 (1954).
3. COHEN, J. A. and WARRINGA, M. G. P. J. Biochim. et Biophys. Acta, **26**, 29 (1957).
4. MAZUR, A. J. Biol. Chem. **164**, 271 (1946).
5. MOUNTER, L. A. J. Biol. Chem. **215**, 705 (1955).
6. MOUNTER, L. A., DIEN, L. T. H., and CHANUTIN, A. J. Biol. Chem. **215**, 691 (1955).
7. GHOSH, R. and NEWMAN, J. F. Chem. & Ind. (London), 118 (1955).
8. HOGEBOOM, G. H., SCHNEIDER, W. C., and PALADE, G. E. J. Biol. Chem. **164**, 271 (1948).
9. PALADE, G. E. and SIEKEWITZ, P. J. Biophys. Biochem. Cytol. **2**, 171 (1956).
10. ROSSITER, R. J. Can. J. Biochem. and Physiol. **35**, 579 (1957).
11. RABINOWITZ, J. L. and Gurin, S. Biochim. et Biophys. Acta, **10**, 345 (1953).
12. MOUNTER, L. A., FLOYD, C. S., and CHANUTIN, A. J. Biol. Chem. **204**, 221 (1953).
13. RACKER, E. and KRINSKY, I. J. Biol. Chem. **161**, 453 (1945).
14. GREEN, D. E., NEEDHAM, D. M., and DEWAN, J. G. Biochem. J. **31**, 2327 (1937).
15. COOPER, J. R. and BRODIE, B. B. J. Pharmacol. Exptl. Therap. **114**, 409 (1955).

SYMPOSIUM ON NEUROSECRETION

This symposium took place on June 11, 1958, at the First Annual Meeting of the Canadian Federation of Biological Societies at Queen's University, Kingston, Ontario. Dr. A. S. V. Burgen, Professor of Physiology at McGill University, acted as Chairman and Editor of the symposium, which was organized by a committee of the Federation under the chairmanship of Dr. W. Kalow, Associate Professor of Pharmacology, University of Toronto.

Dr. MacIntosh was prevented by illness from attending the symposium and his contribution was presented by Dr. Burgen.

INTRODUCTION[1]

A. S. V. Burgen

In introducing this symposium, it is perhaps as well to be clear from the outset what we mean by the term neurosecretion. Neurosecretion may be defined as a production of physiologically active substances within neurons, which substances may act either locally on a neighboring neuron or effector cell or be carried in the blood to act on a distant site. We shall be hearing of both these kinds of action this afternoon. I am sure that some of our speakers will point out that there is probably no fundamental difference between secretion by a neuron and secretion by any other secretory cell.

As originally used by the Scharrers, the term neurosecretory cell was applied to certain cells in the nervous system of insects and the hypothalamico–hyphophyseal system of mammals which showed so-called colloidal droplets or granules. In most cases, characteristic changes occurred on section of the neurons. Proximal to the site of section, the neurons swelled and became loaded with droplets, whereas distal to the section the droplets rapidly disappeared. This observation has been used as evidence that there is a proximodistal movement of material in neurosecretory cells. It still remains unsettled whether these droplets represent the actual hormone secreted by the neurons or some inactive carrier. We have realized since the application of electron microscopy to synaptic structures that these colloidal granules represent only a special case of a more general phenomenon, the presence of microvesicular structures at presynaptic endings. Dr. MacIntosh, in particular, is presenting evidence that these microvesicles represent packages or quanta of humoral transmitter.

Neurosecretion encompasses three distinct problems: (1) the synthesis of the hormone and its enclosing in a microvesicle, (2) the proximodistal migration of the hormone within the neuronal structure, and (3) the release of the hormone from the cell as a result of neuronal activity. Various members of our panel are going to touch on all three aspects of this problem. I would like to say no more about it than to comment that my own feeling is that a solution of many of the mysteries of the last two aspects is going to depend largely on further understanding of the dynamics of the interior membranes

[1]Manuscript received October 20, 1958.
Contribution from the Department of Physiology, McGill University, Montreal, Quebec.

of the cell, that is, the endoplasmic reticulum. Amongst its other functions, it is not unlikely that the endoplasmic reticulum acts as an intracellular transport system.

I cannot close this introduction without drawing the attention of those members of our Federation who have an inflexible attachment to mammals as experimental animals to the very exciting work on neurohormones that has been carried out in invertebrates. For instance, instar formation and metamorphosis in insects is under control of a neurohormonal system composed of a brain stem *x*-organ system and the corpora allata-cardiacum system involving the complex interaction of a molting hormone and a so-called juvenile hormone. The chemistry of both these substances is at an advanced stage of development. Molting in the crustacea is controlled by a neurohormonal system in the eye stalk. The heart rate of crustacea is partly under the control of a local "adrenal", the pericardial organ, a collection of nerve endings in the heart that liberate a cardioaccelerator substance directly into the blood within the heart. These are only a few examples of the fascinating neurohormonal systems that are known to exist in invertebrates. I think those of us who are interested primarily in mammals would be foolish to ignore the contribution in ideas and analogies that invertebrate neurohormonal systems can provide for us.

NEUROHYPOPHYSEAL SECRETION[1]

R. L. NOBLE

The topic for this symposium on neurosecretion would seem a particularly fitting one for this the christening ceremony of the newly born Canadian Federation of Biological Societies since the subject has been a common meeting place of the interests of anatomists, histologists, biochemists, and physiologists. Your chairman asked me to try to blend the findings of these different disciplines into a review emphasizing anatomy and histology which still would be comprehensible to all members of the Federated Societies. References therefore must be general but may be found in the Proceedings of the Eighth Symposium of the Colston Research Society published as The Neurohypophysis (1), The Neural Control of the Pituitary Gland, by G. W. Harris (2), Ham's Histology (3), and the current Annual Review of Physiology (4).

Anatomical Considerations

The gross anatomy of the area surrounding the pituitary gland is too well known to be outlined in detail. A peculiarity of the posterior lobe in certain species, however, is worthy of note since it has frequently been cited by serious-thinking armchair scientists who wish to refute almost any new theory advanced on the hormonal control of certain pituitary functions. In species such as the armadillo, whale, walrus, porpoise, and Indian elephant, the posterior or neural lobe is quite discrete from the anterior and intermediate lobes, from which it may be separated by a thick fibrous septum. Although the rearing of such animals in the laboratory presents certain problems, those that have been studied showed that the pars tuberalis was anterior to the dividing septum and clearly showed the hypophyseal portal system of blood vessels so that no theoretical objections to presently postulated secretory pathways can be considered as valid.

The vascular connections of the pituitary region have received special attention recently and are of key importance to the most recent theory of neurosecretion. Many years ago Professor Rainer of Bucharest commented on the prominent blood vessels running along the pituitary stalk and suggested they should be studied in detail. Later one of his students, Prof. G. T. Popa, working with human material, made serial sections of this region. He concluded that the prominent vessels in reality were portal vessels. They ended in capillaries if traced upwards to the *tuber cinereum* or, if traced downwards, into the hypophysis. With Fielding in 1930 and 1933 these vessels were named the hypothalamo–hypophyseal portal vessels. Unfortunately it was erroneously suggested that blood flowed up these vessels

[1]Manuscript received October 20, 1958.
Contribution from the Department of Medical Research, University of Western Ontario, London, Ontario.

from the pituitary gland to the hypothalamus. Wislocki in a series of papers from 1936 to 1938 added to the older observations but concluded correctly that the blood flowed in the vessels towards the pituitary gland. He also showed that the capillaries at the lower end connected only with the anterior lobe of the pituitary and not with the neural lobe and at the superior end were situated in the infundibular stalk and median eminence of the *tuber cinereum*. Since 1947 Harris and collaborators have described many beautiful preparations of these vessels and have worked out their detailed distribution. Small arterial branches from the internal carotid and posterior communicating arteries join in a rich vascular plexus in the pars tuberalis of the pituitary. From this plexus a multitude of capillary tufts penetrate into the median eminence. There they come into intimate contact with the nerve fibers of the supraopticohypophyseal, tuberohypophyseal, and other nerve tracts. Blood from this primary plexus is drained down the large portal trunks, lying visibly on the surface of the pituitary stalk. The portal trunks in turn break up and distribute their blood into the sinusoids of the pars distalis of the adenohypophysis. It may be noted that Harris has demonstrated that the hypophyseal portal vessels have marked powers of regeneration after experimental section of the pituitary stalk if no impervious type of plate is inserted at the operation.

Histological Considerations

From the time of the earliest observations on the pituitary gland attempts have been made to ascribe to it some type of secretory activity. The long list of erroneous theories is impressive and starts with the naming of the gland by Vesalius in 1543 as the pituitary, indicating the function of secreting mucus into the nose. Willis in 1684, however, believed that the gland really secreted the cerebrospinal fluid, but Magendie in 1847, adopted the converse view that the gland took up the cerebrospinal fluid and secreted it into the blood. Theories concerning secretory activity have continued to come and go with surprising frequency so that there is a veritable graveyard of once attractive bodies of evidence which have now been buried under the present-day theory of neurosecretion. Perhaps I should say nearly buried since even in textbooks of physiology some theories seem to die hard. In retrospect one is led to conclude that the more modern histologist was no doubt impressed by the rapidly unfolding panorama of each new hormone being attributed, shortly after its discovery, to a particular secretory type of cell; in the posterior pituitary, however, he was faced with demonstrable hormones in a tissue devoid of apparent secretory elements and so was tempted to speculate unwisely on the possible cells which might give rise to the hormones.

Most of the immense amount of time-consuming work by histologists and neurologists has concerned the tracing of nerve pathways from nuclei in the hypothalamus to the posterior lobe of the pituitary and has centered on the control of secretion of the posterior lobe hormones. The influence

on the antidiuretic or pressor hormone has been particularly suitable since its lack can be recorded in the intact animal, even by one without advanced training in physiology, as the continuous secretion of great quantities of unconcentrated urine.

Herring Bodies

The earliest of the more recent theories of origin of the posterior lobe hormones was that they were secreted by the cells of the intermediate lobe of the pituitary. It was appreciated by Biedl and others that in many species it was virtually impossible to separate pars intermedia cells from the pars nervosa. Here then, mixed with nervous tissue, were cells of epithelial origin which might secrete hormones. Herring in 1908 postulated that basophile cells of the pars intermedia migrated into the neurohypophysis and became transformed into hyaline masses. These "Herring bodies" were supposed to travel up the pituitary stalk into the cerebrospinal fluid in the third ventricle and then affect nerve centers in the hypothalamus. Such a theory was supported by Harvey Cushing in 1932, who demonstrated apparent parasympathetic activation in patients when posterior lobe extract was injected into the lateral ventricle of the brain. Purified hormones of the posterior lobe, however, do not cause this reaction and detectable amounts of the hormones have not been found in cerebrospinal fluid. Geiling finally drove the nails into the coffin of the pars intermedia theory by demonstrating a full hormone complement in the posterior lobe of some of the unwieldy species already referred to in which this is quite separate and uncontaminated by cells of other lobes of the pituitary. And so the migratory bodies of Herring were discarded into the bone pile of broken theories. Posthumously, however, a footnote must be added—Herring bodies have been resurrected by the champions of the neurohypophyseal secretion theory and apparently do represent depots of stored posterior lobe hormone.

Pituicytes

Besides Herring bodies the pars nervosa contains special types of neuroglial cells which were termed pituicytes by Bucy 1930–1932. As a result of his studies he postulated that these cells were responsible for the formation of the posterior lobe hormones. This theory was quite widely accepted and was supported by reports that in tissue culture antidiuretic hormone was produced by pituicytes after all nerve fibers had degenerated. Gersh in 1939 described granules in these cells which he believed varied with the physiological state such as dehydration of the animal. He further postulated that the nerve fibers which run between the diencephalon and the neural lobe regulated the release of the antidiuretic hormone. The extensive and careful studies by Ranson and colleagues (1938, 1939) apparently supported this theory. In painstaking work, bilateral destruction of the supraoptic and paraventricular nuclei of the hypothalamus was inevitably followed, after an interval, by *diabetes insipidus* and a disappearance of all hormones from the posterior

pituitary gland. They attributed these changes to destruction of the secretomotor innervation of the pituicytes. Histological observation of large spherical inclusions in the nuclei of the pituicytes were also interpreted as evidence of a secretory function.

Death of the pituicyte theory was a more gradual phenomenon and did not occur suddenly—a few still seem to feel that a feeble pulse is discernible. The in vitro experiments however were questioned. If the pituicyte required secretomotor innervation from the hypothalamus to produce hormones, how did it continue to function in culture after degeneration of all nerve fibers? With longer times of culture, although the pituicytes survived, they now did not produce hormones. Hild and others, from morphological studies, claimed the now accepted view that the pituicytes are not secretory cells but rather protoplasmic astrocytes modified by their situation.

Neurosecretion

With the elimination of the Herring body and the pituicyte as the source or origin of the posterior lobe hormones, the conscientious histologist was left with only the thick network of fine unmyelinated nerve fibers of the posterior lobe. He was also faced with the necessity to discard the older concepts of epithelial hormone secretory cells, secretory granules, etc. Histologists such as Scharrer, Gaupp, and others had discussed for many years the possibility that certain nerve cells might secrete hormones into the blood stream. The fine unmyelinated terminations of the supraoptico-hypophyseal tract in the posterior lobe, which stain with silver to show nodular thickenings and form a fine meshed plexus in the vicinity of blood vessels, seemed eminently suited for a neurosecretory function. The development of new staining and histochemical techniques such as the chrome haematoxylin staining method of Gomori and Bargmann, the thioglycollate – ferric ferricyanide reaction for cystine, and others showed a selective capacity to outline the supraoptic and paraventricular nuclei and nerve tracts running from these to the neurohypophysis. Zetler and Hild, Bargmann, and others have now demonstrated selectively staining neurosecretory substance within the neuroplasm. Throughout the posterior lobe the nerve fibers are characterized by beaded thickenings resembling a string of pearls. Large swellings on neurosecretory fibers can be identified as Herring bodies. In these studies, which seem outstanding for use of the bizarre experimental animal, is an important observation by Bodian on the opossum. I cannot refrain at this time from digressing to point out just what an opportunity this problem has been for the comparative anatomist and histologist. At the Colston symposium after hearing presentations on the species previously referred to and in addition presentations on the hormone content of the camel and desert rat, Dr. Hanstrom in discussing lobulated Herring bodies observed by Dr. Bargmann quoted his own similar findings in wolves. He ended by stating that "bulb-shaped endings are outstanding in some mammals". To Sir Henry Dale's question of "what species are you speaking about?" Professor Hanstrom replied "The tiger"!

To return to the opossum, in this species the pars neuralis is curiously lobulated by distinct septa containing numerous small blood vessels. At the periphery of the lobule the terminals of the nerve fibrils can be seen to be coated with a wrapping of neurosecretory substance and to be in immediate contact with the vessels of the septum. Whether the neurosecretory material which stains by the Gomori technique represents the actual hormone or a carrier substance is debatable. Lipid solvents may extract the stainable material in the pig (Hild and Zetler 1953) but leave, on assay, a normal hormone content. In the developing chick embryo Wingstrand in 1953 found that antidiuretic activity was demonstrable in the hypothalamus by 10 days but that Gomori-stainable material did not appear until day 13 or 14. On the other hand the ability of the secretory material to react as cystine in staining techniques is of suggestive significance in view of the richness of this amino acid in the pure hormones. The pure hormones, however, do not show the Gomori staining reaction. The neurosecretory theory envisions the posterior lobe hormones as being formed only in the neurosecretory cells of the supraoptic and paraventricular nuclei of the hypothalamus and being transported down the axons of the hypothalamic–hypophyseal tract to the neural lobe—here a storage of the hormone occurs.

This theory explains the early observations of Abel in 1924 and Melville and Hare in 1945, and others, that extracts of the hypothalamic region of the brain may contain antidiuretic hormone activity. Furthermore most observers have found a striking and close parallelism between the amount of neurosecretory material demonstrable histologically and the level of hormone content found on assay. The dog exhibits surprisingly high levels and man very low levels. Stimulation by dehydration results in hormone liberation and the injection of hypertonic sodium chloride solutions also depletes the Gomori-stainable material but this returns when animals are placed on a normal dietary regimen. Following section of the pituitary stalk of the dog the neural lobe exhibits a decrease in the content of both hormonal and neurosecretory material. On the other hand the hypothalamic side of the section shows an increase in both substances. In a summary of the above evidence one can only agree with Harris' conclusion that the "Neurosecretory origin of the posterior lobe hormones remains an attractive hypothesis. It offers a clear explanation for the lack of secretory elements in the neurohypophysis, and for the presence of antidiuretic substance in the hypothalamus."

Hormone Activity of the Neurohypophysis

The hormone activity of the neurosecretion of the posterior lobe has been studied most extensively and may profitably be reviewed briefly before we delve into the more ethereal possibilities of neurosecretions affecting the anterior pituitary. From the classical work of Dr. Vigneaud and his colleagues the two separate posterior lobe hormones are now well recognized. The vasopressive hormone of the hog contains lysine in the place of arginine which is found in the form of the hormone common to most other species.

Vasopressive activity may be measured by its effects on blood pressure, or on the production of antidiuresis in the hydrated animal. The two vasopressins, however, differ in their relative potency as intravenous antidiuretic agents. The oxytocic hormone has questionable physiological significance during parturition but is responsible for milk ejection during lactation. It exhibits a hypotensive action in fowls and in some species under special conditions it may cause a natriuretic effect. The specific actions of the two hormones are not entirely separate, however, as a small overlap in the type of action is found even with the pure hormones. The old question of whether the two hormones can be derived from a larger parent molecule and whether this represents a storage form of the hormones is still unsettled. It now seems clear, however, that in the supraoptic nuclei and even in the posterior lobes of some species the ratio of vasopressin to oxytocic activity may vary. The work of Van Dyke and colleagues in 1955 has shown that the *tuber cinereum*, supraoptic nuclei, and paraventricular nuclei of the dog are all rich in antidiuretic hormone, containing approximately 20% of the total activity when compared with 80% in the posterior lobe. On the other hand the hypothalamic nuclei of the dog contained only 2.3% of oxytocic activity compared to greater than 97% in the posterior lobe. In the camel a quite different distribution and ratio of the two hormones was found. The reason for the relatively greater concentration of oxytocic hormone in the posterior lobe is not known.

The question may be asked, "How is the distribution of the hormones in the hypothalamus and posterior lobe modified by depletion of one or both hormones?" In lactating dogs, Van Dyke has shown a significant fall in oxytocin in the posterior lobe but also a fall to a lesser extent in the vasopressive hormone. The V/O ratio increased significantly. Changes in the hormone content of the hypothalamic nuclei were suggestive but not of significance. Conflicting reports, however, have been made by others on the effects of lactation on hormone content of the pituitary in different species. Other reports on attempts to cause hormone depletion by such methods as dehydration indicate that changes do occur in the posterior lobe. A severe depletion of one hormone is always accompanied by depletion of the other although in most cases more oxytocin than vasopressin appears to be released. It would seem then that both the hormones are liberated endogenously although the stimulus evoking their release appears physiologically to require the release of only one hormone. Unfortunately, accurate methods are not yet available to assay the hormones released into the blood stream.

The physiologist has made many contributions in determining the factors which may act in an afferent role to stimulate the hypothalamus and secretion of posterior lobe hormones. The work on osmotic action culminating in the work of Verney, Pickford, and others describing osmoreceptors sensitive to changes in the osmotic pressure of the blood and controlling antidiuretic activity is well established. The stimulus of suckling or milking has now been established to initiate a reflex contraction of myoepithelial cells in the

mammary gland. The milk-ejection reflex is brought about by the neurosecretion of the oxytocic hormone. The establishment of this theory has been revised by Folley but it is of historical interest that nearly fifty years ago Ott and Scott observed that the milk-ejection reflex could be mimicked by the injection of posterior lobe extracts. The ability of changes in fluid balance in the body or the reflex act of suckling to influence the posterior lobe secretions fits a rational concept of need and demand. Many of the other stimuli, however, which apparently result in the liberation of posterior lobe hormones have no apparent physiological basis. Thus neurohypophyseal stimulation and liberation of posterior lobe hormones have been reported after fainting, emotional stress, painful stimuli, electroconvulsive therapy, haemorrhage, motion sickness, sexual intercourse, and various drugs including acetylcholine, morphine, certain anaesthetics, and nicotine, effective quantities of which may be absorbed by cigarette smoking. Epinephrine conversely has been shown to inhibit the release of antidiuretic substance. Injection of small amounts of acetylcholine or of the anticholinesterase, DFP, into the region of the supraoptic nucleus causes a transient antidiuresis and it has been suggested that the supraoptic nucleus may be analogous to a peripheral autonomic ganglion, in that it possesses a synapse at which acetylcholine is the transmitter and which is stimulated by nicotine.

Anterior Pituitary, ACTH, and Posterior Pituitary Hormones

Many of the stimuli which result in the release of the posterior lobe hormones are identical with those invoking a release of ACTH from the anterior pituitary. With the well-developed portal system connecting the median eminence to the anterior pituitary, as previously discussed, it has been a temptation to physiologists to speculate on the possibility of posterior lobe hormones acting as humoral agents to stimulate the anterior pituitary, particularly as both the adrenal cortex and the posterior pituitary share in the normal physiological control of body fluids. It is now well established that Gomori-stainable material in the neurohypophysis is depleted by noxious stimuli that simultaneously evoke an increased discharge of ACTH—adrenalectomy, pain, restraint and noise, and various other stresses are effective stimuli. In most experiments the release of ACTH has been indicated by its primary action of inducing compensatory adrenal hypertrophy after unilateral adrenalectomy or by its secondary effects due to the release of adrenal glucocorticoids, such as the depletion of adrenal ascorbic acid, induction of a blood eosinopenia, or an increase in blood adrenal steroids. Experimental lesions in the hypothalamus may block the liberation of ACTH in response to certain stimuli but the exact site of effective lesions is not yet established. In some reports anterior hypothalamic lesions have prevented compensatory adrenal hypertrophy and anterior and middle median eminence lesions have blocked the eosinopenic response to stress. Some workers have found in rats that only posterior hypothalamic lesions blocked stimuli expected to cause both ascorbic acid depletion and compensatory adrenal hypertrophy.

Conversely electrical stimulation of the posterior hypothalamus has been followed by activation of the adrenal cortex. Of the various lesions described which block ACTH release some but not all have been followed by *diabetes insipidus* presumably due to an induced deficiency of the antidiuretic hormone. Other studies have concerned the blocking of the ACTH-release response by drugs. Morphine is particularly effective as is treatment with adrenal glucocorticoids. Dibenzyline is probably ineffective.

The activity of the "isolated" pituitary gland is of obvious importance to the problem of whether activation by neurohypophyseal hormones can occur. The interpretation of such studies has been difficult but some stimuli have undoubtedly been shown to cause liberation of ACTH under such conditions. It seems probable that the anterior pituitary, even though isolated from the direct influence of the hypothalamus, may continue to secrete ACTH but at a slow rate and is no longer affected by stimuli acting through a nervous reflex. However, as Fortier has suggested, stimuli acting through the general circulation may still activate the isolated gland. In such a concept effective stimuli may be divided into two types, neural, mediated via the nervous system, or systemic, mediated via the blood stream.

Hormonal Transmitters

Studies on the nature of the hormonal transmitter in the hypophyseal portal vessels are not conclusive. Blood removed from the supposed portal area has been found potent in stimulating ACTH release. Although posterior lobe hormones, particularly vasopressin seem implicated in some experiments, results of most studies to date would argue against posterior lobe hormones being the main hormonal transmitter regulating ACTH release. Recently the more elegant in vitro techniques have been used extensively by Guillemin, Saffran, and others and are particularly pertinent to the problem of the nature of hormonal transmitters. Dr. Saffran no doubt will report his own experiences in the next presentation.

Other Anterior Pituitary Hormone Secretions

Although a great deal of work has been devoted to neurosecretion and the release of the thyrotrophic and gonadotrophic hormones, there is little evidence to implicate the posterior lobe hormones as chemical mediators except perhaps in the case of oxytocin for the gonadotrophins.

Thyrotrophic Hormones

That a close endocrinological relationship exists between the thyroid gland and the adrenal cortex is now well established. In general it may be noted that stressful stimuli which excite the adrenal cortex appear to inhibit the activity of the thyroid gland. Conversely the increased production of TSH is accompanied by reduced adrenal activity. It has been suggested that this reciprocal relationship may obtain at the pituitary level and so be

intimately concerned with neurosecretions. Bilateral lesions in the hypothalamus between the suprachiasmatic and caudal ventromedian nuclei result in thyroid atrophy as first reported by Greer. Many others have confirmed that depressed thyroid function follows lesions in the anterior hypothalamus close to the median eminence. Electrical stimulation of hypothalamic areas has been followed by evidence of thyroid stimulation. On the other hand stimulation in the region of the *tuber cinereum* frequently results in thyroid inhibition. As shown by Harris, however, this may be a secondary depression related to a stimulation of ACTH secretion. Little is known of the neurosecretion which may activate pituitary TSH and there is no suggestion that the posterior lobe hormones are involved.

Gonadotrophic Hormones

The participation of a nervous reflex in the release of the luteinizing gonadotrophic hormone (LH) in species such as the rabbit where ovulation follows copulation or stimulation of the uterine cervix, was established many years ago. Similarly the stimulating effect of light on the gonads of birds was acknowledged. If one accepts the lack of direct innervation of the anterior pituitary then gonadotrophin release must follow anterior pituitary stimulation by either a neurosecretion carried in the pituitary portal system or through the general blood circulation. Although epinephrine applied *in situ* to the anterior pituitary may in some cases cause a release of LH, most evidence suggests that some form of portal neurosecretion occurs. Lesions in the basal *tuber cinereum* or median eminence are followed by gonadal atrophy in both sexes in a number of species. Other lesions in the anterior hypothalamus and mammillary nuclei may eliminate mating behavior. On the other hand it has recently been reported that bilateral lesions in the anterior hypothalamus of female ferrets may be followed by the development of oestrus during the winter months, suggesting the removal of hypothalamic inhibition over anterior pituitary secretion. There is some suggestive evidence that oxytocin may be implicated as a neurosecretion affecting gonadotrophin release. Although it might be logical to suspect oxytocin of a dual role in the release of gonadotrophin and prolactin and in mammary gland function, the evidence is not yet convincing.

Summary

Although the concept of neurosecretion is a relatively new one, it seems supported by convincing evidence in the case of posterior-lobe pituitary hormones. Here specific histological techniques can demonstrate a substance associated with hormone activity, apparently originating in two specific nuclei in the hypothalamus and migrating down nerve fibers to concentrate in the posterior lobe of the pituitary. Secretion of the hormone occurs predominantly into the blood stream.

The well-defined hypothalamic–hypophyseal portal blood supply suggests a direct means of transport of neurosecretions from the neighborhood of the

median eminence to the anterior gland of the pituitary. Of growing interest are the demonstrations that activation of pituitary ACTH, TSH, and gonadotrophins may occur through a neurosecretion from the hypothalamus. Although quite selective regions of the hypothalamus can be implicated from ablation experiments, and seem to differ for each of the involved hormones, there is not yet a histological technique comparable with that used in studies on the posterior pituitary to demonstrate specific neurosecretory material.

There is not conclusive evidence that posterior lobe hormones may act as a neurosecretion to activate the anterior pituitary although the vasopressive hormone and oxytocic hormone have seemed to be implicated in some way in the release of ACTH and gonadotrophins.

It is most probable that the liberation of anterior pituitary hormones from hypothalamic neurosecretions follows stimuli of a neural nature evoking a nervous reflex. The anterior pituitary even when isolated from the hypothalamus, however, may still respond to stimuli of a systemic nature. A dual control of the anterior pituitary may therefore be envisioned in which the neural type of stimulus may evoke a more rapid secretory response.

In conclusion one may perhaps question whether the theory of neurosecretion will join its predecessors, neatly arranged in rows, the mounds over each marked with a cross. Perhaps, however, an element of immortality can be detected in this new theory and we can look forward to a new era where neurosecretions will fall logically into place in the scheme of homeostasis. To each hormone, specific functions, with one form of control through individual centers in the hypothalamus. Here afferent stimuli result in sending out through the nerve fibrils separate secretions, each a different chemical entity which after a voyage through the portal system stimulates the release of a specific anterior lobe hormone.

References

1. The neurohypophysis. Proceedings of the eighth symposium of the Colston Research Society, University of Bristol. *Edited by* H. S. Heller. Butterworth Scientific Publications, London. 1957.
2. HARRIS, G. W. Neural control of the pituitary gland. Edward Arnold & Co., London. 1955.
3. HAM, A. W. Histology. J. B. Lippincott Co., Philadelphia. 1953.
4. SAYERS, G., REDGATE, E. S., and ROYCE, P. C. Ann. Rev. Physiol. **21**, 243 (1958).

ACTIVATION OF ACTH RELEASE BY NEUROHYPOPHYSIAL PEPTIDES[1]

MURRAY SAFFRAN

Introduction

Control of Adenohypophysis

The proximity of the pituitary body to the brain led the earliest anatomists to suggest that the pituitary was under the influence of the brain (1). They visualized the gland as a funnel by which the *pituita* or slime of the brain was led into the nose. The *pituita* was considered to consist of the waste product of the change, which occurred in the ventricles of the brain, of vital into animal spirits. Nowadays, the pituitary gland is still believed to be influenced by the brain, but in a way at the same time more complicated and more subtle than that imagined by 16th century anatomists.

There is no difficulty in considering that the neurohypophysis is under nervous control, as the neurohypophysis is actually a part of the brain, and is connected to other parts of the brain by well-defined nerve tracts. However, in spite of claims to the contrary (2), there seem to be no important nervous connections to the adenohypophysis (3). On the other hand, there does exist a network of fine blood vessels in the hypothalamic area at the base of the brain that coalesces into larger vessels leading down the pituitary stalk from the hypothalamus to the pituitary gland, where they once more break up to serve the blood sinuses in the adenohypophysis. This portal circulation, first described by Popa and Fielding in 1930 (4), is ideally suited to carry products of the hypothalamus directly to the adenohypophysis, and is the keystone of the theory that humoral agents from the hypothalamus control the function of the adenohypophysis (3). Thus modern theory is strikingly similar to that of the ancients.

The function of the adenohypophysis is to synthesize, store, and release a variety of peptidic hormones, which have profound influence on metabolism and function. These include ACTH (adrenocorticotrop(h)ic hormone, corticotrop(h)in), TSH (thyroid-stimulating hormone, thyrotrop(h)in), STH (somatotrop(h)in, growth hormone), the various gonadotrop(h)ins, and, although this is not a popular idea today, perhaps other powerful hormonal agents. Each adenohypophysial hormone is presumably controlled by a specific hypothalamic humor, elaborated in response to appropriate nervous

[1]Manuscript received October 20, 1958.
Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. Supported by a Federal–Provincial Mental Health grant No. 604-5-45, and by a grant from the Foundations' Fund for Research in Psychiatry, both to Dr. R. A. Cleghorn; and with generous financial and material support provided by N. V. Organon, Oss, Holland; Canada Packers Limited, Toronto; Parke, Davis and Company, Detroit; and Nordic Biochemicals Limited, Montreal. We are indebted to Dr. A. V. Schally, Dr. R. Guillemin, Dr. V. Du Vigneaud, and Sandoz (Canada) Limited for gifts of valuable posterior pituitary hormone preparations. The original work described in this communication was carried out with the able assistance of Miss B. U. Zimmermann and Miss P. J. Ward.

stimuli. More attention has been given recently to the study of the control of ACTH release than to the other hormones, probably because the mechanism is rapid, responding in seconds in contrast with the hours and days of the other hormones, and because convenient methods are available to detect the release of ACTH. The primary function of ACTH is to stimulate the adrenal cortex, and the release of ACTH results in signs of adrenocortical stimulation, such as adrenal hypertrophy, depletion of adrenal ascorbic acid and lipids, increased formation of adrenocortical steroids, and the subsequent effects of the adrenal hormones on blood cells, on the lymphatic system, and on metabolism.

Chemical Nature of Hypothalamic Products

The so-called posterior pituitary hormones, vasopressin and oxytocin, are probably manufactured in the neurones comprising the paraventricular and supraoptic nuclei of the hypothalamus, and are transported endoaxonally to the nerve endings in the neurohypophysis (Scharrer and Scharrer (5)). Oxytocin and vasopressin are cyclic octapeptides, of strikingly similar composition and structure (6). Also present in the hypothalamus in higher concentrations than in other parts of the brain are the potent agents, adrenaline and noradrenaline (7), histamine (8), serotonin (9), and substance P (9). All but the last named are amines derived from cyclic amino acids, while substance P is probably a peptide. Unlike vasopressin and oxytocin, these substances have not been demonstrated to be secreted into the blood to act on a specific target organ. The neurohypophysis also contains a protein, called neurophysine (10), which can be isolated in combination with one equivalent each of vasopressin and oxytocin, but from which these neurohypophysial hormones can be removed by relatively mild treatment. Neurophysine, even with the hormones removed, gives the same staining reactions as the neurosecretory material found in the hypothalamus and neurohypophysis (11). Thus, the hypothalamo–neurohypophysial region of the brain can manufacture chemical mediators from amino acids, ranging in complexity from amines to proteins. This repertoire does not rule out the possibility that carbohydrate or lipid derivatives may also be converted to neurohumoral agents, but the suggestions for the nature of the ACTH mediator, with a single exception (12), have been that it is a derivative of amino acids.

Antidiuretic Hormone and ACTH Release

The principal biological activity of vasopressin is its intense antidiuretic action on the kidney, hence the term antidiuretic hormone, or ADH. The antidiuretic effect is exerted at doses many times smaller than the amounts required to produce an increase in blood pressure. ADH, therefore, has two known target tissues, the kidney and the vasculature, with different sensitivities. A third biological activity of ADH has often been suggested: the stimulation of ACTH release from the adenohypophysis. The suggestive evidence ranges from the anatomical continuity of the hypothalamus and the neurohypophysis, to the more direct demonstration of ACTH release by

ADH in animals rendered insensitive to ACTH release by other stimuli through operation or treatment with drugs. Only some of the evidence will be cited here.

The proposal that the hypothalamus is involved in the control of ACTH release led to statements that, because the hypothalamus and neurohypophysis are continuous, the controlling agent would be concentrated, like vasopressin and oxytocin, in the neurohypophysis (13). The release of ACTH from the pituitary is usually associated with stimuli, collectively known as stressors, that threaten the homeostatic equilibrium of the animal. Such stressors were observed to deplete the stainable neurosecretory material in the neurohypophysis, suggesting an association between the posterior pituitary hormones, vasopressin and oxytocin (hitherto postulated to be concerned with the neurosecretory material), and the release of ACTH (14). In addition, the blood of rats exposed to stressors contained increased antidiuretic activity, although this was not proved to be due to increased antidiuretic hormone (15). Finally, the administration of preparations of antidiuretic hormone caused signs of the release of ACTH in normal animals (16,17,18) and in animals bearing hypothalamic lesions that abolished the ACTH release that normally follows the stimulus of a stressor (19). However, two groups of investigators independently suggested that the ACTH-releasing activity of the preparations of antidiuretic hormone was in reality ascribable to a distinct peptide present as an impurity (20, 21). The active material, termed "corticotropin-releasing factor" or CRF, was purified and found to be similar in amino acid composition to the antidiuretic hormone (22) and to possess significant antidiuretic activity itself (21,23). In view of the similarity in composition and of the overlap in antidiuretic activity, antidiuretic hormone and oxytocin were re-examined for ACTH-releasing activity.

Detection of CRF

The isolation of CRF was made possible by an in vitro test which depends upon the stimulation of the release of ACTH from isolated rat adenohypophysial tissue incubated in oxygenated Krebs–Ringer–bicarbonate medium containing glucose (24). A rat anterior pituitary is divided in two, and the halves are incubated separately. If nothing is added to either half, the two pieces of the same gland release the same amount of ACTH (21,24–26). The addition of CRF-containing samples to one half results in the release of more ACTH than is released by the control piece to which nothing is added. As pointed out by Fortier (26, 27) and by Sayers (28), apparent stimulation of ACTH release can also occur (*a*) if the added sample contains ACTH, (*b*) if the added sample potentiates the action of ACTH in the assay method for ACTH (29), (*c*) if the added sample protects ACTH leaving the pituitary tissue from inactivation, (*d*) if the added sample actually does stimulate ACTH release, or (*e*) if a combination of these events occurs. Obviously, before describing experiments utilizing the in vitro test for ACTH-releasing activity, it is necessary to show that the test is valid.

median eminence to the anterior gland of the pituitary. Of growing interest are the demonstrations that activation of pituitary ACTH, TSH, and gonadotrophins may occur through a neurosecretion from the hypothalamus. Although quite selective regions of the hypothalamus can be implicated from ablation experiments, and seem to differ for each of the involved hormones, there is not yet a histological technique comparable with that used in studies on the posterior pituitary to demonstrate specific neurosecretory material.

There is not conclusive evidence that posterior lobe hormones may act as a neurosecretion to activate the anterior pituitary although the vasopressive hormone and oxytocic hormone have seemed to be implicated in some way in the release of ACTH and gonadotrophins.

It is most probable that the liberation of anterior pituitary hormones from hypothalamic neurosecretions follows stimuli of a neural nature evoking a nervous reflex. The anterior pituitary even when isolated from the hypothalamus, however, may still respond to stimuli of a systemic nature. A dual control of the anterior pituitary may therefore be envisioned in which the neural type of stimulus may evoke a more rapid secretory response.

In conclusion one may perhaps question whether the theory of neurosecretion will join its predecessors, neatly arranged in rows, the mounds over each marked with a cross. Perhaps, however, an element of immortality can be detected in this new theory and we can look forward to a new era where neurosecretions will fall logically into place in the scheme of homeostasis. To each hormone, specific functions, with one form of control through individual centers in the hypothalamus. Here afferent stimuli result in sending out through the nerve fibrils separate secretions, each a different chemical entity which after a voyage through the portal system stimulates the release of a specific anterior lobe hormone.

References

1. The neurohypophysis. Proceedings of the eighth symposium of the Colston Research Society, University of Bristol. *Edited by* H. S. Heller. Butterworth Scientific Publications, London. 1957.
2. HARRIS, G. W. Neural control of the pituitary gland. Edward Arnold & Co., London. 1955.
3. HAM, A. W. Histology. J. B. Lippincott Co., Philadelphia. 1953.
4. SAYERS, G., REDGATE, E. S., and ROYCE, P. C. Ann. Rev. Physiol. **21**, 243 (1958).

ACTIVATION OF ACTH RELEASE BY NEUROHYPOPHYSIAL PEPTIDES[1]

MURRAY SAFFRAN

Introduction

Control of Adenohypophysis

The proximity of the pituitary body to the brain led the earliest anatomists to suggest that the pituitary was under the influence of the brain (1). They visualized the gland as a funnel by which the *pituita* or slime of the brain was led into the nose. The *pituita* was considered to consist of the waste product of the change, which occurred in the ventricles of the brain, of vital into animal spirits. Nowadays, the pituitary gland is still believed to be influenced by the brain, but in a way at the same time more complicated and more subtle than that imagined by 16th century anatomists.

There is no difficulty in considering that the neurohypophysis is under nervous control, as the neurohypophysis is actually a part of the brain, and is connected to other parts of the brain by well-defined nerve tracts. However, in spite of claims to the contrary (2), there seem to be no important nervous connections to the adenohypophysis (3). On the other hand, there does exist a network of fine blood vessels in the hypothalamic area at the base of the brain that coalesces into larger vessels leading down the pituitary stalk from the hypothalamus to the pituitary gland, where they once more break up to serve the blood sinuses in the adenohypophysis. This portal circulation, first described by Popa and Fielding in 1930 (4), is ideally suited to carry products of the hypothalamus directly to the adenohypophysis, and is the keystone of the theory that humoral agents from the hypothalamus control the function of the adenohypophysis (3). Thus modern theory is strikingly similar to that of the ancients.

The function of the adenohypophysis is to synthesize, store, and release a variety of peptidic hormones, which have profound influence on metabolism and function. These include ACTH (adrenocorticotrop(h)ic hormone, corticotrop(h)in), TSH (thyroid-stimulating hormone, thyrotrop(h)in), STH (somatotrop(h)in, growth hormone), the various gonadotrop(h)ins, and, although this is not a popular idea today, perhaps other powerful hormonal agents. Each adenohypophysial hormone is presumably controlled by a specific hypothalamic humor, elaborated in response to appropriate nervous

[1]Manuscript received October 20, 1958.
Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. Supported by a Federal–Provincial Mental Health grant No. 604-5-45, and by a grant from the Foundations' Fund for Research in Psychiatry, both to Dr. R. A. Cleghorn; and with generous financial and material support provided by N. V. Organon, Oss, Holland; Canada Packers Limited, Toronto; Parke, Davis and Company, Detroit; and Nordic Biochemicals Limited, Montreal. We are indebted to Dr. A. V. Schally, Dr. R. Guillemin, Dr. V. Du Vigneaud, and Sandoz (Canada) Limited for gifts of valuable posterior pituitary hormone preparations. The original work described in this communication was carried out with the able assistance of Miss B. U. Zimmermann and Miss P. J. Ward.

stimuli. More attention has been given recently to the study of the control of ACTH release than to the other hormones, probably because the mechanism is rapid, responding in seconds in contrast with the hours and days of the other hormones, and because convenient methods are available to detect the release of ACTH. The primary function of ACTH is to stimulate the adrenal cortex, and the release of ACTH results in signs of adrenocortical stimulation, such as adrenal hypertrophy, depletion of adrenal ascorbic acid and lipids, increased formation of adrenocortical steroids, and the subsequent effects of the adrenal hormones on blood cells, on the lymphatic system, and on metabolism.

Chemical Nature of Hypothalamic Products

The so-called posterior pituitary hormones, vasopressin and oxytocin, are probably manufactured in the neurones comprising the paraventricular and supraoptic nuclei of the hypothalamus, and are transported endoaxonally to the nerve endings in the neurohypophysis (Scharrer and Scharrer (5)). Oxytocin and vasopressin are cyclic octapeptides, of strikingly similar composition and structure (6). Also present in the hypothalamus in higher concentrations than in other parts of the brain are the potent agents, adrenaline and noradrenaline (7), histamine (8), serotonin (9), and substance P (9). All but the last named are amines derived from cyclic amino acids, while substance P is probably a peptide. Unlike vasopressin and oxytocin, these substances have not been demonstrated to be secreted into the blood to act on a specific target organ. The neurohypophysis also contains a protein, called neurophysine (10), which can be isolated in combination with one equivalent each of vasopressin and oxytocin, but from which these neurohypophysial hormones can be removed by relatively mild treatment. Neurophysine, even with the hormones removed, gives the same staining reactions as the neurosecretory material found in the hypothalamus and neurohypophysis (11). Thus, the hypothalamo–neurohypophysial region of the brain can manufacture chemical mediators from amino acids, ranging in complexity from amines to proteins. This repertoire does not rule out the possibility that carbohydrate or lipid derivatives may also be converted to neurohumoral agents, but the suggestions for the nature of the ACTH mediator, with a single exception (12), have been that it is a derivative of amino acids.

Antidiuretic Hormone and ACTH Release

The principal biological activity of vasopressin is its intense antidiuretic action on the kidney, hence the term antidiuretic hormone, or ADH. The antidiuretic effect is exerted at doses many times smaller than the amounts required to produce an increase in blood pressure. ADH, therefore, has two known target tissues, the kidney and the vasculature, with different sensitivities. A third biological activity of ADH has often been suggested: the stimulation of ACTH release from the adenohypophysis. The suggestive evidence ranges from the anatomical continuity of the hypothalamus and the neurohypophysis, to the more direct demonstration of ACTH release by

ADH in animals rendered insensitive to ACTH release by other stimuli through operation or treatment with drugs. Only some of the evidence will be cited here.

The proposal that the hypothalamus is involved in the control of ACTH release led to statements that, because the hypothalamus and neurohypophysis are continuous, the controlling agent would be concentrated, like vasopressin and oxytocin, in the neurohypophysis (13). The release of ACTH from the pituitary is usually associated with stimuli, collectively known as stressors, that threaten the homeostatic equilibrium of the animal. Such stressors were observed to deplete the stainable neurosecretory material in the neurohypophysis, suggesting an association between the posterior pituitary hormones, vasopressin and oxytocin (hitherto postulated to be concerned with the neurosecretory material), and the release of ACTH (14). In addition, the blood of rats exposed to stressors contained increased antidiuretic activity, although this was not proved to be due to increased antidiuretic hormone (15). Finally, the administration of preparations of antidiuretic hormone caused signs of the release of ACTH in normal animals (16,17,18) and in animals bearing hypothalamic lesions that abolished the ACTH release that normally follows the stimulus of a stressor (19). However, two groups of investigators independently suggested that the ACTH-releasing activity of the preparations of antidiuretic hormone was in reality ascribable to a distinct peptide present as an impurity (20, 21). The active material, termed "corticotropin-releasing factor" or CRF, was purified and found to be similar in amino acid composition to the antidiuretic hormone (22) and to possess significant antidiuretic activity itself (21,23). In view of the similarity in composition and of the overlap in antidiuretic activity, antidiuretic hormone and oxytocin were re-examined for ACTH-releasing activity.

Detection of CRF

The isolation of CRF was made possible by an in vitro test which depends upon the stimulation of the release of ACTH from isolated rat adenohypophysial tissue incubated in oxygenated Krebs–Ringer–bicarbonate medium containing glucose (24). A rat anterior pituitary is divided in two, and the halves are incubated separately. If nothing is added to either half, the two pieces of the same gland release the same amount of ACTH (21,24–26). The addition of CRF-containing samples to one half results in the release of more ACTH than is released by the control piece to which nothing is added. As pointed out by Fortier (26, 27) and by Sayers (28), apparent stimulation of ACTH release can also occur (*a*) if the added sample contains ACTH, (*b*) if the added sample potentiates the action of ACTH in the assay method for ACTH (29), (*c*) if the added sample protects ACTH leaving the pituitary tissue from inactivation, (*d*) if the added sample actually does stimulate ACTH release, or (*e*) if a combination of these events occurs. Obviously, before describing experiments utilizing the in vitro test for ACTH-releasing activity, it is necessary to show that the test is valid.

Results

Experiments on Validity of Test

(*a*) *Presence of ACTH in Samples*

The presence of ACTH in the samples tested can be detected by direct assay for ACTH, or more simply taken into account by adding the test material to the medium from the control pituitary tissue at the end of the incubation. These precautions were taken as a matter of routine. For example, Fig. 1 illustrates the effect of adding various test extracts to adrenals in the ACTH bio-assay system. Only the relatively enormous dose of 100 μg of crude CRF gave a significantly positive test for ACTH. This contamination can then be reckoned with in further tests with crude CRF. In the doses used, neither Pitressin nor the synthetic arginine vasopressin exerted any ACTH-like effect on rat adrenals, while in the presence of pituitary tissue, the release of ACTH (Fig. 1) was dependent upon the dose of the material added. Thus the presence of ACTH in the test solutions did not explain the apparent stimulation of ACTH release (cf. 21).

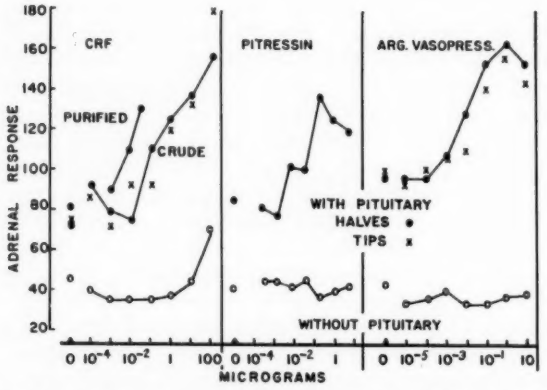


FIG. 1. The in vitro adrenal response (formation of Δ^4-3-ketosteroids) to neurohypophysial materials alone (○) and after incubation with rat anterior pituitary tissue (●, using pituitary halves; ×, using only the outer tips of the pituitary gland).

(*b*) *Potentiation of ACTH Action*

Neither we nor Guillemin *et al.* (21) could detect any potentiating effect of materials used *in the doses tested* in the assay for ACTH.

(*c*) *Protection of ACTH*

Barrett and Sayers (28) are convinced that the demonstration of ACTH-releasing activity in vitro is explicable in terms of the protection of ACTH against destruction by peptidases. Fortier, too, is inclined to favor this explanation, based, apparently, on a single experiment in which he found a

40% destruction (measured by our in vitro assay (29)) of U.S.P. standard ACTH by incubation, without tissue, for 1 hour (27). Barrett and Sayers (28) base their conviction on many experiments using either 8-hour-long incubations of whole rat pituitary glands or 1-hour incubations of rat pituitary homogenates, using the adrenal ascorbic acid assay for ACTH (30). Under these conditions, widely differing from those of the in vitro ACTH-release test, pituitary ACTH is rapidly destroyed by the pituitary peptidases liberated either during the course of the 8-hour incubation or by disruption of cellular structure. The ACTH is protected against destruction under these conditions by the addition of basic peptides, such as pitressin or glucagon. On the other hand, Barrett and Sayers themselves state that there is no detectable decline in ACTH activity during the course of a 30-minute incubation period with whole pituitaries, which is more comparable with the 60-minute period used in the ACTH-release test.

Barrett and Sayers go on to suggest that the ideal test to settle the question is as follows: "Medium separated from halved pituitaries after 10 minutes of incubation may be divided into two aliquots. To one aliquot add 'CRF' or glucagon and to the other make no addition. Incubate both samples for 1 hour. Equal quantities of ACTH in the control and test samples would support the 'release' concept; less ACTH in the control than in the sample to which 'CRF' had been added would support the 'inhibition of destruction' concept." (28). Together with Dr. A. V. Schally, we have carried out this experiment, the only difference being that the initial incubation was 1 hour, instead of 10 minutes. The results of three experiments (Table I) showed that there were equal quantities of ACTH in the control and test samples, thus supporting the "release" concept. Moreover, under our conditions of incubation and assay, added ACTH is not detectably destroyed, either in incubation medium alone, or with pituitary tissue (Fig. 2).

TABLE I

Effect of purified CRF on recovery of "native" rat ACTH after aerobic incubation at 38° for 1 hour in Krebs–Ringer–bicarbonate–glucose medium at pH 7.4

Expt. No.	Recovery, %	95% limits, %
1	107	74–154
2	90	64–126
3	81	53–125
Geometric mean	92	74–114

(d) Stimulation of ACTH Release

Therefore, the only remaining explanation for our results is that our test actually does detect stimulation of the release of ACTH from rat pituitary tissue in vitro.

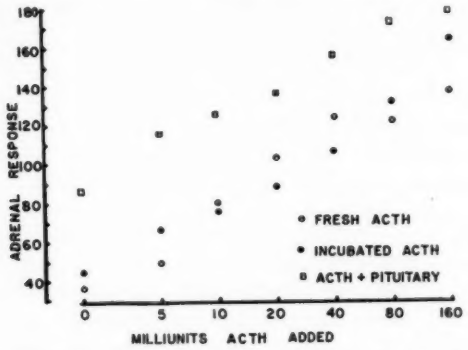


FIG. 2. Adrenal response to incubated and fresh U.S.P. standard ACTH.

ACTH RELEASE BY NEUROHYPOPHYSIAL MATERIALS

Time Curve of ACTH Release

The test period of 1 hour (24) was originally chosen arbitrarily. Figure 3 illustrates that the control tissue releases relatively little ACTH in this period, whereas crude CRF causes a rapid and steady release of ACTH. In longer incubation periods (Fig. 4), the control tissue continues its slow release for about 2 hours, after which it levels off or may decline, perhaps because of destruction of the released ACTH by peptidases. The stimulated tissue continues to release augmented amounts of ACTH for a long time, although the rate falls off after the first hour. The choice of 1 hour for the incubation period is therefore justified.

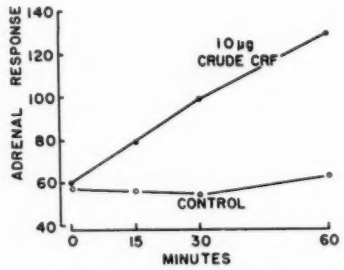


FIG. 3. Release of ACTH by rat adenohypophysial tissue incubated with and without CRF, during the course of 1 hour.

Anaerobiosis

No stimulation of ACTH release by pitressin or synthetic arginine vasopressin occurred in an atmosphere of N_2–CO_2 (Fig. 5). The release with crude CRF was erratic, but there was no evidence of the relatively smooth dose–response curve seen in aerobic incubations.

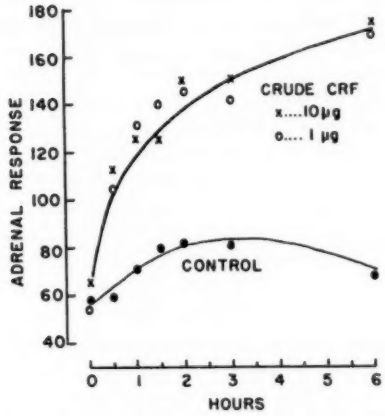


FIG. 4. Release of ACTH during the course of 6 hours' incubation.

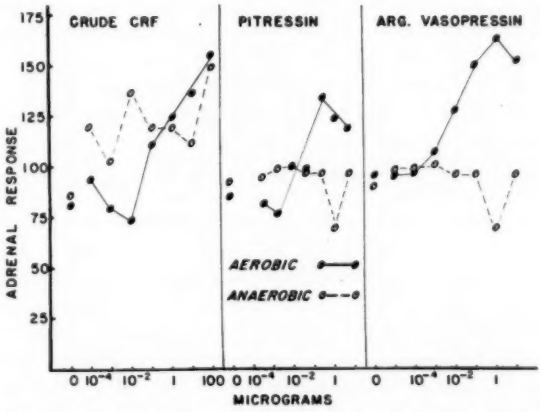


FIG. 5. Release of ACTH aerobically (95% O_2 – 5% CO_2) and anaerobically (95% N_2 – 5% CO_2).

Size of Pituitary Sections

Practically identical dose–response curves were obtained with pituitary halves and with smaller pieces of tissue obtained by snipping off the tips of the glands distal from the neurohypophysis. Thus, contrary to Barrett and Sayers' (28) suggestion that cutting the tissue could influence the leaching of peptidases from the wound, the size of the tissue sections, and hence, the relative tissue damage, did not influence the response to the added material.

Purification of CRF

Purified CRF, prepared by Dr. A. V. Schally to the second *m*-cresol stage (22), gave the same slope as the crude preparation in the dose–response line, but at lower dose levels (Fig. 1).

Comparing the Various Preparations

Figure 6A illustrates the dose–response relationship, in the ACTH-release test, of synthetic oxytocin (Syntocin, Sandoz), crude CRF (CRF-91, Organon), purified CRF (Schally, second *m*-cresol stage), Pitressin (Parke, Davis preparation of vasopressin), lysine vasopressin (isolated from posterior pituitary extract by Ward and Guillemin (31)), and synthetic arginine vasopressin (Du Vigneaud). The slopes of the curves are strikingly similar, and on the basis of the weight of material, range in potency from lysine vasopressin to oxytocin. The purified CRF and synthetic arginine vasopressin are almost as active as the lysine vasopressin. It should be noted here that the purified

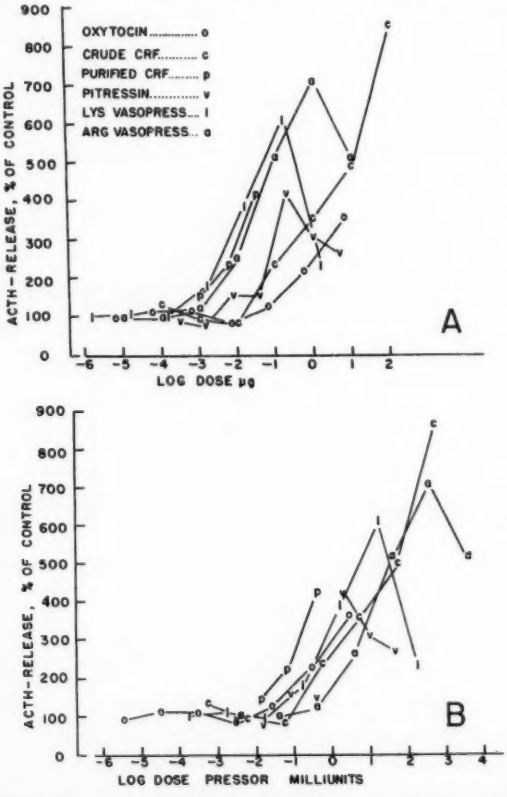


FIG. 6. ACTH release by neurohypophysial preparations: (A) Dose plotted by weight. (B) Dose plotted by pressor activity.

CRF is only 1/30–1/50 as pure as the best CRF preparation (22). When the dose is plotted in terms of pressor activity, the preparations align themselves differently (Fig. 6B). Now arginine vasopressin is the least potent, the purified CRF is most potent, while the other preparations have more or less equal activity. The vasopressin preparations are distinguished by an apparent decrease in ACTH release after attaining a maximum effect. Such a fall-off did not occur with the crude CRF, even when the significant ACTH contamination at the highest dose level was accounted for. Unfortunately the purified CRF was not tested at elevated dose levels. However, Guillemin (21) reported a similar fall-off with high doses of his purified ACTH-releasing peptide.

Discussion

All the neurohypophysial preparations tested exhibit activity in the in vitro ACTH-release test. Oxytocin is the least active, and purified CRF and the vasopressins are the most active. On the basis of previous experience, still purer preparations of CRF would be even more active. Former doubts about the activity of lysine vasopressin in this test (25) can be explained by the previous use of elevated doses, which fell on the descending limb of the dose–response curve.

In view of the chemical resemblance of the vasopressins, oxytocin, and CRF it is not surprising that they share certain biological properties. Thus, vasopressin is oxytoxic, with about 5% the activity of oxytocin; CRF is antidiuretic, with about 10% the activity of the antidiuretic hormone (23); and lysine vasopressin releases ACTH, with about 10% the activity of highly purified CRF (this paper, 22). The ACTH-releasing activity of vasopressin explains the results of the investigators who believe it to be the corticotropin-releasing factor (16–19). On the other hand, the antidiuretic activity of CRF may explain the increased antidiuretic titer in the blood of animals exposed to stress (15).

Under certain circumstances it is possible to dissociate the release of antidiuretic hormone and the release of ACTH. It has long been known that a large load of water inhibits the secretion of the antidiuretic hormone, otherwise the water could not be excreted. Yet, a large load of water, while inhibiting ADH release, acts as a stressor and causes increased release of ACTH (32–34). This dissociation has understandably raised doubts about the obligatory participation of ADH in ACTH release.

At the same time evidence is accumulating favoring the role of CRF in the control of ACTH. Guillemin's group have given their purified material to human subjects and noted increases in blood corticoids (35), while Rochefort (36) found that CRF administration produced sizeable reductions in pituitary stores of ACTH, even in animals whose response to stress is blocked by previous treatment with cortisol. CRF also enhanced the incorporation of P^{32} into the phospholipids of anterior pituitary tissue (37), a phenomenon that apparently accompanies the stimulation of the release, by a specific stimulant, of stored peptidic materials (38).

There is nothing unusual in an endocrine organ producing a family of chemically related hormones with overlapping biological properties. The adrenal cortex produces cortisol, corticosterone, and aldosterone. These three corticoids differ merely by the disposition of a hydroxyl group; yet, while sharing each other's properties, they exhibit different major biological activities. The hypothalamus–neurohypophysis seems to produce a family of related peptides, with actions on different target tissues: vasopressin acts mainly on the kidney; oxytocin, on the mammary glands and uterus; CRF, on the adenohypophysis. Other activities have also been postulated to be formed by this part of the brain (reviewed in 39), with the eye (40), voluntary muscle (41), the capillaries (42), and the liver (43) as target tissues.

Summary

The anatomical relationship of the adenohypophysis to the hypothalamico–neurohypophysial area of the brain has long suggested a controlling influence of this part of the brain on adenohypophysial function. Three peptide materials found in the neurohypophysis, vasopressin, oxytocin, and corticotropin-releasing factor (which may be products of the system producing the stainable neurosecretory material), were examined for their ability to stimulate the release of ACTH from adenohypophysial tissue in vitro. All three kinds of preparations were active, with oxytocin exhibiting the least activity. Evidence for the validity of the test system is presented, along with a discussion of the evidence favoring vasopressin or CRF as an obligatory intermediary in the release of ACTH in response to stress. The suggestion is made that the ability to promote the release of ACTH is a property shared by a family of substances, rather than a property of a unique neurohormone.

References

1. CUSHING, H. Pituitary body, hypothalamus and parasympathetic nervous system. Charles C. Thomas, Publisher, Springfield, Ill. 1932.
2. METUZALS, J. J. Endocrinol. **14**, 87 (1956).
3. HARRIS, G. W. Neural control of the pituitary gland. Edward Arnold & Co., London. 1955.
4. POPA, G. T. and FIELDING, U. J. Anat. **65**, 88 (1930).
5. SCHARRER, E. and SCHARRER, B. Recent Progr. in Hormone Research, **10**, 183 (1954).
6. DU VIGNEAUD, V. Harvey Lectures, Ser. L, (1954–1955).
7. VOGT, M. J. Physiol. **123**, 451 (1954).
8. HARRIS, G. W., JACOBSOHN, D., and KAHLSON, G. Ciba Foundation Coll. Endocrinol. **4**, 186 (1954).
9. AMIN, A. A., CRAWFORD, T. B. B., and GADDUM, J. H. J. Physiol. **126**, 596 (1954).
10. ACHER, R., CHAUVET, J., and OLIVRY, G. Biochim. et Biophys. Acta, **22**, 421 (1956).
11. ACHER, R. Zweites internationales Symposium über Neurosekretion, Lund, Sweden. *Edited by* W. Bargmann, B. Hanström, B. Scharrer, and E. Scharrer. Springer-Verlag, Berlin. 1958. p. 71.
12. SLUSHER, M. A. and ROBERTS, S. Endocrinology, **55**, 245 (1954).
13. ZUCKERMAN, S. Ciba Foundation Coll. Endocrinol. **4**, 213 (1952).
14. ROTHBALLER, A. B. Anat. Record, **115**, 21 (1953).
15. MIRSKY, I. A., STEIN, M., and PAULISCH, G. Endocrinology, **54**, 491 (1954).
16. MARTINI, L. and MORPURGO, C. Nature, **175**, 1127 (1955).
17. SOBEL, H., LEVY, R. S., MARMORSTON, J., SCHAPIRO, S., and ROSENFELD, S. Proc. Soc. Exptl. Biol. Med. **89**, 10 (1955).
18. MCDONALD, R. K., WEISE, V., and PATRICK, R. W. Proc. Soc. Exptl. Biol. Med. **93**, 348 (1956).

19. McCann, S. M. and Brobeck, J. R. Proc. Soc. Exptl. Biol. Med. **87**, 318 (1954).
20. Saffran, M., Schally, A. V., and Benfey, B. G. Endocrinology, **57**, 439 (1955).
21. Guillemin, R., Hearn, W. R., Cheek, W. R., and Housholder, D. E. Endocrinology, **60**, 488 (1957).
22. Schally, A. V., Saffran, M., and Zimmermann, B. U. Biochem. J. **70**, 97 (1958).
23. Segal, M. Rev. can. biol. **16**, 513 (1957); Saffran, M., Schally, A. V., Segal, M., and Zimmermann, B. U. Zweites internationales Symposium über Neurosekretion, Lund, Sweden. *Edited by* W. Bargmann, B. Hanström, B. Scharrer, and E. Scharrer. Springer-Verlag, Berlin. 1958. p. 55.
24. Saffran, M. and Schally, A. V. Can. J. Biochem. and Physiol. **33**, 408 (1955).
25. Schally, A. V. and Saffran, M. Proc. Soc. Exptl. Biol. Med. **92**, 636 (1956).
26. Fortier, C. Texas Repts. Biol. and Med. **16**, 68 (1958).
27. Fortier, C. and Ward, D. N. Can. J. Biochem. and Physiol. **36**, 111 (1958).
28. Barrett, A. M. and Sayers, G. Endocrinology, **62**, 637 (1958).
29. Saffran, M. and Schally, A. V. Endocrinology, **56**, 523 (1955).
30. Sayers, M. A., Sayers, G., and Woodbury, L. A. Endocrinology, **42**, 379 (1948).
31. Ward, D. N. and Guillemin, R. Proc. Soc. Exptl. Biol. Med. **96**, 568 (1957).
32. Nagareda, C. S. and Gaunt, R. Endocrinology, **48**, 560 (1951).
33. Schapiro, S., Marmorston, J., and Sobel, H. Endocrinology, **62**, 278 (1958).
34. McDonald, R. K., Wagner, H. N., Jr., and Weise, V. K. Proc. Soc. Exptl. Biol. Med. **96**, 652 (1957).
35. Clayton, G. W., Bell, W. R., and Guillemin, R. Proc. Soc. Exptl. Biol. Med. **96**, 777 (1957).
36. Rochefort, G. J. Ph. D. Thesis. McGill University, Montreal. 1958; Rochefort, G. J., Rosenberger, J., and Saffran, M. J. Physiol. In press. (1959).
37. Hokin, M. R., Hokin, L. E., Saffran, M., Schally, A. V., and Zimmermann, B. U. J. Biol. Chem. **233**, 811 (1958).
38. Hokin, L. E. and Hokin, M. R. Biochim. et Biophys. Acta, **16**, 229 (1955).
39. Saffran, M. Ergeb. inn. Med. u. Kinderheilk. **10**, 594 (1958).
40. Dietz, A. A., Schmerl, E., and Steinberg, B. Arch. Biochem. **70**, 1 (1957).
41. Ingle, D. J. and Li, C. H. Endocrinology, **57**, 383 (1955).
42. Kramar, J., Meyers, W. V., McCarthy, H. H., Dietz, N., Jr., Simay-Kramar, M., and Williams, J. W. Am. J. Physiol. **188**, 387 (1957).
43. Seifter, J. and Baeder, D. H. Proc. Soc. Exptl. Biol. Med. **95**, 318 (1957).

ADRENERGIC MEDIATORS[1]

MARK NICKERSON

Knowledge of the adrenergic mediators extends over a period of more than 60 years. Adrenaline was the first hormone discovered, and it was also the first to be isolated, identified chemically, and synthesized. More than 50 years ago, long before acetylcholine was identified as a normal constituent of the animal body, Elliott (1) suggested that adrenaline might be ". . . the chemical stimulant liberated on each occasion when the [sympathetic nerve] impulse arrives at the periphery." However, despite this long history and the fact that the adrenergic mediators are more stable than is acetylcholine, present knowledge of adrenergic neurohumoral transmission is much less definitive than is knowledge of the peripheral cholinergic system. Almost all aspects of adrenergic transmission, from synthesis of the neurohumors to their ultimate destruction, are still clouded with unanswered questions and unresolved conflicts of observation and opinion. This presentation will attempt to outline briefly current knowledge relating to adrenergic neurohumors, with particular emphasis on its limitations, and to formulate certain major problems in this field.

The Adrenergic Mediators

Adrenaline has by far the longest history of any of the candidates for the designation "adrenergic mediator." It is found in large amounts in the adrenal medulla of most species of animals, and its release into the blood stream undoubtedly plays a role in many responses which involve this organ. It is generally accepted that release of adrenal medullary hormones into the blood stream is an important component of certain metabolic responses which involve the sympatho-adrenal system, such as the response to hypoglycemia (2, 3), but the adrenal medulla appears to play only a minor role in cardiovascular adjustments (4). Most cardiovascular and other smooth muscle responses are activated primarily by mediator released locally from adrenergic nerve endings, and adrenaline never has been shown unequivocally to play a role in the action of these nerves. As early as 1910 Barger and Dale (5) pointed out that the pharmacodynamic characteristics of a primary amine such as noradrenaline more closely parallel the actions of adrenergic nerves than do those of a secondary amine such as adrenaline. Since that time various workers sporadically have called attention to the characteristics of noradrenaline which would qualify it as the major sympathetic nerve mediator.

[1]Manuscript received October 20, 1958.
Contribution from the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba. Presented as part of a Symposium on Neurosecretion at the first annual meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, June 11, 1958. Work from the author's laboratory included in this paper has been supported by grants-in-aid from the National Research Council of Canada, the Burroughs Wellcome Co., and Ayerst, McKenna and Harrison, Ltd.

However, this substance was not shown to be present in the animal body until 1946 (see 6). Since that time the work of Euler and of many others has established noradrenaline to be the major adrenergic constituent of sympathetic nerves and to be present in varying amounts in the adrenal medulla. It is now generally accepted that noradrenaline is a major mediator of adrenergic nerve activity, but it cannot be said with any assurance that it is *the mediator*.

I feel it is dangerous to ignore completely the older observations of Cannon and Rosenblueth (7) which indicated that the characteristics of the mediator escaping into the blood stream after stimulation of sympathetic nerves to different organs could vary. Adrenaline and noradrenaline are contained in separate cells in the adrenal medulla, but it is not clear whether the small amount of adrenaline found in many nerves is a minor constituent of most of the nerve fibers or the major constituent of a few. It is conceivable that preferential release of adrenaline might allow it to play a more important role in the action of some nerves than their relative content of this amine would indicate, but no direct evidence in support of this possibility has been adduced.

Although adrenaline and noradrenaline have received major attention, they are not the only candidates for the title of adrenergic mediator. It has been reported that a substance clearly distinguishable from adrenaline or noradrenaline, both pharmacologically and chromatographically, comprises 80 to 100% of the adrenergic material released into the blood stream during stimulation of the pulmonary sympathetic nerves in the cat heart–lung preparation (8). The agent could not be differentiated from isopropylnoradrenaline (isoproterenol, isoprenaline) by the technics employed, but clear differentiation between the isopropyl and other higher N-alkyl derivatives of noradrenaline has not been accomplished. A similar material also has been obtained in small amounts from the adrenal glands of several species, including man (9), but its physiological significance is still unclear. It is plausible that if the final step in the synthesis of adrenaline is simply N-methylation of noradrenaline (*vide infra*), alkyl substituents larger than methyl also might be introduced.

Synthesis of Adrenergic Mediators

The synthesis of adrenergic mediators is much more complex than the simple acetylation of choline to produce acetylcholine. The available data have been obtained from studies on the adrenal medulla rather than on adrenergic nerves, but it is assumed that the processes in nerve are similar. No convincing evidence for the synthesis of adrenergic mediators in tissues other than adrenergic nerves and chromaffin cells has been presented. This localization of synthesis is indicated by the marked drop in catechol amine content of most organs after denervation (6, 10, 11).

Tracer studies have clearly established that adrenaline and noradrenaline can be produced from both tyrosine and phenylalanine (12), the latter

probably via tyrosine. The biosynthesis of adrenaline from tyrosine would require four distinct steps: phenolic hydroxylation, decarboxylation, alkyl hydroxylation, and N-methylation (Fig. 1). It is probable that the steps are carried out in this order, as originally suggested by Blaschko (13), but the possibility that introduction of the alkyl hydroxyl group precedes decarboxylation, producing dihydroxyphenylserine as an intermediate, cannot be excluded entirely.

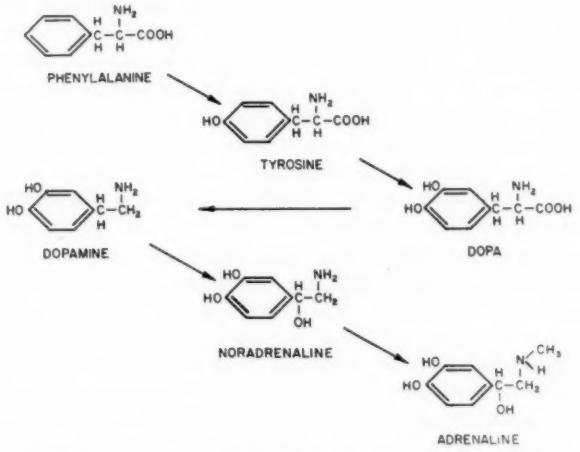


FIG. 1. Probable sequence of steps in the synthesis of adrenaline from phenylalanine (see text for details).

Knowledge of the mechanisms by which hydroxyl groups are added to the aromatic ring is very limited. Tyramine does not appear to be involved in the synthesis of the catechol amines (14), and, consequently, oxidation to form dihydroxyphenylalanine (dopa) appears to be an essential first step in the transformation of tyrosine. This reaction can be catalyzed by tyrosinase, an enzyme which has been demonstrated in several tissues, particularly skin, but not in the adrenal medulla. It is possible that dopa is transferred to the adrenal from other sites or that it is formed nonenzymatically. Its production from tyrosine at pH 7.0 in the presence of ascorbic acid and oxygen has been demonstrated in vitro (15), an observation which focuses attention on the very high ascorbic acid content of the adrenal. Decarboxylation of dopa and subsequent steps in the synthesis probably occur more rapidly than does ring hydroxylation. Administration of dopa, but not of phenylalanine or tyrosine, can significantly increase the rate of reaccumulation of adrenaline in the adrenal medulla of the rat partially depleted by insulin-induced hypoglycemia (16).

The enzyme required for the next step, dopa decarboxylase, is present in the adrenal medulla in large amounts (17, 18), and its product, dopamine, has been identified in adrenal glands (11) and in other tissues and urine. Many

attempts to demonstrate dopamine in the adrenals of various species have been unsuccessful, but its extraction is complicated by the fact that it is rapidly deaminated by monoamine oxidase, which also is present in large amounts. For this reason, positive results probably should be given more weight than negative observations. Dopamine is a much better substrate for monoamine oxidase than are adrenaline and noradrenaline, and it is probable that most of the dihydroxyphenylacetic acid found in both free and conjugated forms in urine (19) is derived from this source and, consequently, is not directly related to the metabolism of adrenaline and noradrenaline.

Although introduction of the alkyl hydroxyl group is known to occur in the adrenal, its position in the sequence of synthesis has been unclear. Although decarboxylation of dihydroxyphenylserine is much slower than decarboxylation of dopa, it can occur in vivo (20, 21). Hagen (22) has provided the most convincing evidence for a two-step conversion of dopa to noradrenaline via dopamine by incubating dopa-C^{14} with a particle-free supernatant fraction from ox adrenal medulla (free of monoamine oxidase), isolating and characterizing the resultant dopamine-C^{14}, and then incubating this with a homogenate of chicken adrenal to produce labelled noradrenaline. Few details of the reaction leading to the alkyl hydroxylation of dopamine are known, but the enzyme system involved is of particular importance because it controls the isomeric specificity of the synthesis. Hagen found that bioassay and radiochemical and spectrophotometric determinations of the yield of noradrenaline from dopamine gave almost identical results, indicating that only the *l*-isomer was formed.

The methyl group of adrenaline is derived at least in part from methionine in vivo (23). Methylation probably is the last step in the formation of adrenaline. Older work suggested that adrenaline increased when minced adrenal tissue was incubated with noradrenaline and ATP (24) (demethylation appeared to occur if ATP was not added) and during perfusion of the dog adrenal *in situ* (25). It has been noted also that the noradrenaline content of adrenal glands (10, 26) and of adrenaline-containing granules (27) may increase during the early period of presumably increased synthesis associated with insulin hypoglycemia. More recently, conversion of noradrenaline-C^{14} to adrenaline-C^{14} has been demonstrated in vivo (28), and Kirshner and Goodall (29) have characterized and partially purified an enzyme, noradrenaline methylpherase, from the nonparticulate cytoplasm of adrenal medulla which actively methylates noradrenaline with S-adenosylmethionine as the immediate methyl donor.

However, the possibility of a synthetic reaction bypassing noradrenaline by the N-methylation of dopamine has not been excluded. It is improbable that formation of N-methyldopa is involved in the synthesis because no system for the decarboxylation of N-methylamino acids is known. It is of interest to note that the system involved in N-methylation is the last to develop during ontogeny. Fetal adrenals and those from newborn animals

contain large amounts of noradrenaline, but very little adrenaline, even in species in which the latter is the major component of the adult adrenal medulla (10, 30).

Recent histochemical observations (31, 32) have demonstrated that the adrenal medulla contains two types of cells, one containing predominantly adrenaline and the other noradrenaline. The relative numbers of these two types of cells agree well with the chemically determined ratio of the two catechol amines in various species. This observation strongly suggests that methylation of noradrenaline is limited to certain cells, a very specific type of biochemical differentiation between cells which have been considered to be morphologically identical.

A still indistinct relation between the production of adrenaline and the adrenal cortex has been suggested by several observations. In general, the percentage of adrenaline found in the adrenal medulla varies directly with the cortex/medulla ratio when various species are compared, and during ontogeny the percentage of adrenaline increases roughly parallel to the development of the cortex (30). It has been noted also that hypophysectomy decreases and administration of corticotropin (ACTH) increases the percentage of adrenaline (10) and that the percentage of adrenaline in the adrenal medulla of a patient with Addison's disease was abnormally low (33). Finally, in certain reptiles where the medulla lies largely on the surface of the cortex, adrenaline is found only in the small tongues of medullary cells which penetrate into the cortex (34). The physiological significance of these observations is unclear, but together they suggest some functional relationship.

The adrenergic mediators appear to be synthesized relatively more slowly than is acetylcholine. The adrenal medulla can be depleted of its catechol amines by protracted stimulation of secretion, particularly in response to hypoglycemia, and repletion may require several days, although there is considerable unexplained variation among the observations of different workers (10, 12, 16, 26). However, repletion is a direct measure of storage rather than of synthesis, and much of the variability may be due to variations in secretion rate under different experimental conditions. Isotope studies in which tyrosine-C^{14} or phenylalanine-C^{14} was administered to label the adrenaline and noradrenaline in adrenals with a normal catechol amine content provided a figure of 9 days for the half-life of the adrenal catechol amines in the rat, and a secretion rate of 0.002 to 0.004 μg per kg per minute (12). However, this rate is 20 to 100 times less than the directly determined values obtained by other workers in larger laboratory animals (35, 36) and is even lower than the rate of catechol amine secretion by completely denervated cat adrenals (37). In the tracer experiments, the tyrosine pool of the body retained a considerable amount of C^{14} for extended periods, which would contribute to a spuriously long half-life for the adrenal catechol amines.

At least a large part of the adrenaline and noradrenaline in the adrenal medulla is stored in small (0.1 to 0.6 μ) cytoplasmic bodies which are similar to but probably not identical with mitochondria (38). They are not "secretory

granules", and may be mitochondria in which much of the other material has been displaced by the accumulation of catechol amines. The relatively small amounts of adrenaline and noradrenaline found in the supernatant appear always to be in the same ratio as in the granules, suggesting that they may have been released during preparation. It is of interest that both dopa decarboxylase and noradrenaline methylpherase, enzymes clearly involved in the biosynthesis of the catechol amines, are found in the nonparticulate cytoplasm, indicating that the mediators are transferred to granules for storage rather than being synthesized within granules.

The catechol amine content in these granules is extremely high, up to 19% of their dry weight and about 0.6 mg/mg of protein (39). The content of individual granules appears to be predominantly, if not entirely, either adrenaline or noradrenaline (27). The active amines are loosely bound within the granules and are readily released by relatively mild procedures such as altered pH or tonicity. It is possible that ATP, which appears to be accumulated in granules with the catechol amines in a fairly constant molar ratio (40), is involved in the accumulation and binding.

Release of Adrenergic Mediators

Very little is known regarding the intimate mechanism of the release of adrenergic mediators. Acetylcholine from preganglionic fibers is the initiating physiological stimulus in the adrenal medulla (41) and a variety of agents with nicotinic properties appear to act similarly. Mediators at sympathetic nerve endings normally are released by the arrival of a nerve impulse which probably does not involve chemical mediation. Release at this locus has been quantitated only roughly, but the available data suggest that the amount per impulse is approximately constant in splenic nerves up to a frequency of 30 to 50/second and falls to very low values at frequencies > 100/second (42).

The depolarization and associated permeability changes of sympathetic adrenergic nerve endings and probably of medullary cells, which are embryologically and physiologically equivalent to postganglionic sympathetic neurones, could allow the release of catechol amines from the cytoplasm by simple diffusion. However, the problem becomes more complex if all of the active material is contained in granules. Acetylcholine ordinarily does not readily penetrate cells, and does not cause release from granules in vitro (38). All currently available data are compatible with the assumption that depolarization causes the release of a limited amount of mediator which is free in the cytoplasm, and that this labile store is replenished from the intracellular granules. However, presently available techniques do not appear to provide any way of checking this assumption, or of determining unequivocally whether any adrenaline or noradrenaline is free in the cytoplasm of adrenal medullary cells or of sympathetic postganglionic nerves.

One of the more interesting aspects of the physiology of adrenergic mediators is the relatively selective release of adrenaline or noradrenaline by various indirect stimuli. Quantitation of this phenomenon is to a considerable

extent limited by inadequacies of the biological and chemical methods available for the determination of these amines. However, certain effects are of sufficient magnitude and appear to be sufficiently consistent to establish the principle of differential release. The most thoroughly studied response is that to insulin-induced hypoglycemia, which involves a predominant release of adrenaline (see 6). In the cat, a species in which the adrenal medulla contains roughly equal amounts of adrenaline and noradrenaline, the release of adrenaline is affected much more than that of noradrenaline by changes in blood glucose concentration, and this relation is abolished by denervation (35). These observations indicate a selective innervation of the two types of medullary cells.

Experiments reported by Folkow and Euler (36) provide particularly clear-cut evidence for the selective release of both adrenaline and noradrenaline from cat adrenals. These investigators assayed the catechol amine output in adrenal vein blood during periods of stimulation through electrodes introduced into the hypothalamus, and found areas in which stimulation caused predominantly a release of adrenaline and others in which the induced catechol amine secretion was largely noradrenaline. These data do not yet allow any precise correlation with functional areas in the brain stem, but it is reasonable to assume that functional relations will be demonstrated.

In addition to more physiological mechanisms of release of adrenaline and noradrenaline, it is known that reserpine and other *Rauwolfia* alkaloids can cause a rapid release of adrenergic mediators from both sympathetic nerves and adrenal medulla (43, 44). This action is accompanied by signs of increased adrenergic activity, followed by a more prolonged period during which responses characteristic of adrenergic nerve activity are reduced or absent. No direct evidence that reserpine interferes with the synthesis of catechol amines has been presented, but the chronic decrease in adrenergic responses suggests that the over-all turnover of catechol amines as well as storage may be decreased. Release of adrenergic mediators in response to motor nerve stimulation is not inhibited by botulinum toxin, as is the release of acetylcholine (45), nor by the classical adrenergic blocking agents (46). However, it has been reported that choline 2:6 xylyl ether can decrease or abolish responses to sympathetic nerve stimulation and the release of noradrenaline from splenic nerves without inhibiting responses to exogenous mediators or transmission in sympathetic nerve trunks (47). It has been postulated, but not proved, that this agent interferes with some step in the synthesis of adrenaline and noradrenaline.

Mechanism of Action of Adrenergic Mediators

The released adrenergic mediators are assumed to produce their effects by reacting with receptors in or on susceptible cells. These receptors are of at least two types: α-receptors, involved in the stimulation of smooth muscle and of some exocrine glands, and β-receptors, involved in the relaxation of most smooth muscle and in cardiac stimulation (48). This classification is

only roughly parallel to the older designations of E (excitatory) and I (inhibitory) responses. Adrenaline is somewhat more effective than is noradrenaline in activating α-receptor responses, and is very much more effective in activating most β-receptors. With increasing size of the N-alkyl group, predilection for β-receptors is increased and isopropylnoradrenaline (isoproterenol, isoprenaline) produces predominantly β-receptor responses. There is considerable overlap in the ability of various sympathomimetic amines to activate α- and β-receptors, but blocking agents more clearly distinguish between them. The classical adrenergic blocking agents such as Dibenamine, the ergot alkaloids, etc., are effective only in blocking α-receptors (49), whereas 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (Lilly 20522, the dichloro analogue of isopropylnoradrenaline) appears to have equal specificity for β-receptors (50).

Among the possible adrenergic mediators, differences in ability to produce α- and β-responses appear to be due primarily to differences in "affinity" for the specific receptors rather than to differences in ability to activate the receptors after combination has occurred ("intrinsic activity"). Although isoproterenol is approximately 1000 times less effective than is adrenaline in stimulating vascular smooth muscle, the stimulation produced per receptor occupied is essentially the same for the two compounds (51). Combination with the tissue receptors usually is considered to be transient, but interference with other reactions of the receptors suggests that the stimulant may be present in some, perhaps altered, form long after its obvious effects have been dissipated (52). Many aspects of the activation of receptors by neurohumoral agents are unclear, and interpretation of experimental observations has been further complicated by the recent demonstration that a maximal tissue response may be elicited by occupancy of a relatively small percentage of the total receptors (53, 54).

The normal rates of discharge of sympathetic neurones, even under conditions of maximal reflex excitation, are relatively low, not greater than 6 to 8/second (55), and at these frequencies most of the response is local. Escape of excess mediator into the blood stream, and consequent effects on more distant effectors are appreciable only at frequencies of 10/second and above (4, 42, 55).

Disposition of Adrenergic Mediators

The mechanisms by which the body terminates the action of adrenergic mediators, not necessarily the same as their ultimate fate or destruction, still are not fully understood. Many pathways of destruction and inactivation have been investigated and shown to be possible. However, none has yet been demonstrated to play a decisive role such as that of the cholinesterases in cholinergic transmission. The monoamine oxidase found in most tissues has received intensive investigation as the "cholinesterase" of the adrenergic system. Tracer studies have demonstrated that about 50% of large doses of exogenously administered adrenaline may be excreted as deaminated metabolites by the rat, and this fraction is sharply reduced by the administration of

an amine oxidase inhibitor (56). However, adrenaline and noradrenaline are relatively poor substrates for this enzyme, probably because of the presence of the alcoholic hydroxyl group, and essentially complete inhibition of monoamine oxidase usually does not potentiate their actions, increase the percentage excreted in free form (57), or alter the amount of noradrenaline found in the effluent blood during stimulation of the splenic nerves (42). It must be concluded that present evidence supplies little support for any important role of monoamine oxidase in adrenergic neurohumoral transmission.

Oxidation of the phenolic hydroxyl groups with the production of adrenochrome or noradrenochrome and ultimately melanins is another possible pathway. These reactions occur spontaneously in vitro and are catalyzed by heavy metal ions. However, adrenochrome never has been shown to be formed in vivo and its production during the metabolism of adrenaline is highly improbable (56, 58). It is interesting to note that lack of evidence for the natural occurrence of adrenochrome has not inhibited speculation regarding its role in a variety of physiological and pathological processes.

Conjugation of catechol amines to form inactive sulphate esters or glucuronides has been demonstrated to occur in vivo, but the most convincing evidence for this mechanism derives from experiments in which very large doses of adrenaline were administered orally (58, 59). Conjugation of many substances occurs in the intestinal wall and liver, and it is questionable whether these reactions are of specific significance in the normal disposition of catechol amines. Normal urine contains a small amount of adrenaline and noradrenaline in an inactive form which can be "activated" by acid hydrolysis. These physiologically inactive materials presumably are conjugates of the catechol amines, but their nature and significance have not been investigated.

Recent identification of 3-methoxy-4-hydroxymandelic acid as a metabolite of noradrenaline in human urine (60) and of an enzyme system catalyzing 3-methylation of catechol amines (61) has again focused attention on the phenolic hydroxyl groups as a site of inactivation. However, nothing is known regarding the rapidity of this reaction in vivo or of its possible association with sites of release of adrenergic mediators, and no evidence for a physiological role of this pathway in the termination of the action of adrenergic mediators has been presented.

In contrast to cholinergic effects, the decay of responses to adrenergic mediators is sufficiently slow to allow diffusion to be a factor in their termination. It frequently has been shown that exogenous adrenaline can be accumulated in cells in large amounts both in vitro (62) and in vivo (63, 64), and that at least in the case of erythrocytes, it is relatively stable in this environment. It appears quite possible that simple diffusion into the blood stream and ultimately into a large volume of intracellular water may be a significant factor in terminating adrenergic responses, particularly when large amounts of mediator are released. Dawes (65) has presented experimental evidence suggesting that certain amidine and guanidine derivatives may potentiate the action of adrenaline administered into the portal circulation by inhibiting its penetration into liver cells. The possibility of "inactivation" by simple

diffusion complicates the interpretation of all attempts to define the loci of destruction of the catechol amines on the basis of infusion into specific vascular beds.

It has long been known that adrenaline and noradrenaline produce little systemic effect when injected intra-arterially into the systemic circulation (66), but it is obvious that these amines readily pass unchanged through the pulmonary arterial bed where their vasoconstrictor action is much weaker. This difference has been assumed to be due to the marked reduction in blood flow induced in the systemic vascular beds and the resultant extended period for inactivation. However, another interpretation of these observations is possible. One could assume that the adrenergic mediators are inactivated in the process of producing their effect, i.e., that the receptors involved in the response are also the agency of inactivation. Brown and Gillespie (42) recently have presented cogent evidence in support of this possibility. These investigators assayed the noradrenaline in the splenic venous blood during and after stimulation of the splenic nerves in cats, and found that none was detectable at physiological stimulation rates of < 10/second. The noradrenaline per stimulus then increased sharply to a maximum at 30 to 50/second. Prior administration of Dibenamine or phenoxybenzamine (Dibenzyline) to block the α-receptors allowed recovery of the maximal noradrenaline per stimulus at all frequencies from 1 to 50/second. These results can best be explained on the assumption that the receptors themselves inactivate the mediator, this representing the major pathway of inactivation until saturated at high frequencies of nerve stimulation. The time course of the decay of responses to sympathetic nerve stimulation also suggests that diffusion from the immediate site is of importance in terminating the action only at rates of stimulation above the physiological range (55). It appears that the statement made by Elliott in 1905 (66) that ". . . adrenaline disappears in the tissues which it excites" may have previously unappreciated significance.

On the basis of currently available information one can conceive of a sequence of three mechanisms in the disposition of adrenergic mediators. (1) Inactivation by combination with receptors may be the most important process under physiological conditions, but many aspects of this mechanism are as yet unexplored. (2) When the amounts of mediator are in excess of those required to saturate the receptor system, diffusion into the blood stream and into inert intracellular loci may terminate the action. (3) Enzymatic processes involving deamination and/or conjugation may provide for the final, relatively slow disposal of mediator accumulated in intracellular water and perhaps of some mediator very slowly released from receptors without inactivation.

References

1. ELLIOTT, T. R. J. Physiol. **31**, XX (1904).
2. CANNON, W. B., MCIVER, M. A., and BLISS, S. W. Am. J. Physiol. **69**, 46 (1924).
3. HOUSSAY, B. A., MOLINELLI, E. A., and LEWIS, J. T. Rev. asoc. méd. arg. **37**, 486 (1924).
4. CELANDER, O. Acta Physiol. Scand. **32**, Suppl. 116 (1954).
5. BARGER, G. and DALE, H. H. J. Physiol. **41**, 19 (1910).

6. EULER, U. S. VON. Noradrenaline: chemistry, physiology, pharmacology, and clinical aspects. Charles C. Thomas, Springfield, Ill. 1956.
7. CANNON, W. B. and ROSENBLUETH, A. Am. J. Physiol. **104**, 557 (1933).
8. LOCKETT, M. F. Brit. J. Pharmacol. **12**, 86 (1957).
9. LOCKETT, M. F. Brit. J. Pharmacol. **9**, 498 (1954).
10. HÖKFELT, B. Acta Physiol. Scand. **25**, Suppl. 92 (1951).
11. GOODALL, McC. Acta Physiol. Scand. **24**, Suppl. 85 (1951).
12. UDENFRIEND, S., COOPER, J. R., CLARK, C. T., and BAER, J. E. Science, **117**, 663 (1953).
13. BLASCHKO, H. J. Physiol. **101**, 337 (1942).
14. GOODALL, McC. and KIRSHNER, N. J. Biol. Chem. **226**, 213 (1957).
15. VAN ARMAN, C. G. and JONES, K. K. J. Invest. Dermatol. **12**, 11 (1949).
16. VAN ARMAN, C. G. Am. J. Physiol. **164**, 476 (1951).
17. LANGEMANN, H. Brit. J. Pharmacol. **6**, 318 (1951).
18. HOLTZ, P. and BACHMANN, F. Naturwissenschaften, **39**, 116 (1952).
19. EULER, C. VON, EULER, U. S. VON, and FLODING, I. Acta Physiol. Scand. **33**, Suppl. 118, 32 (1955).
20. BEYER, K. H. Biosynthesis and metabolism of phenylethyl (pressor) amines. *In* Chemical factors in hypertension, Advances in chemistry series. No. 2. American Chemical Society, Washington. 1950. p. 37.
21. BLASCHKO, H., BURN, J. H., and LANGEMANN, H. Brit. J. Pharmacol. **5**, 431 (1950).
22. HAGEN, P. and WELCH, A. D. Recent Progr. in Hormone Research, **12**, 27 (1956).
23. KELLER, E. B., BOISSONNAS, R. A., and DU VIGNEAUD, V. J. Biol. Chem. **183**, 627 (1950).
24. BÜLBRING, E. Brit. J. Pharmacol. **4**, 234 (1949).
25. BÜLBRING, E. and BURN, J. H. Brit. J. Pharmacol. **4**, 245 (1949).
26. WEST, G. B. Brit. J. Pharmacol. **6**, 289 (1951).
27. SCHÜMANN, H. J. J. Physiol. **137**, 318 (1957).
28. MASUOKA, D. T., SCHOTT, H. F., AKAWIE, R. I., and CLARK, W. G. Proc. Soc. Exptl. Biol. Med. **93**, 5 (1956).
29. KIRSHNER, N. and GOODALL, McC. Biochim. et Biophys. Acta, **24**, 658 (1957).
30. SHEPHERD, D. M. and WEST, G. B. Brit. J. Pharmacol. **6**, 665 (1951).
31. ERÄNKÖ, O. Nature, **175**, 88 (1955).
32. HILLARP, N.-A. and HÖKFELT, B. Acta Physiol. Scand. **30**, 55 (1953).
33. WEST, G. B., SHEPHERD, D. M., and HUNTER, R. B. Lancet, **2**, 966 (1951).
34. WRIGHT, A. and JONES, I. C. Nature, **175**, 1001 (1955).
35. DUNER, H. Acta Physiol. Scand. **28**, Suppl. 102 (1953).
36. FOLKOW, B. and EULER, U. S. VON. Circulation Research, **2**, 191 (1954).
37. VOGT, M. Brit. J. Pharmacol. **7**, 325 (1952).
38. BLASCHKO, H., HAGEN, P., and WELCH, A. D. J. Physiol. **129**, 27 (1955).
39. HILLARP, N.-A. and NILSON, B. Acta Physiol. Scand. **32**, 11 (1954).
40. BLASCHKO, H., BORN, G. V., D'IORIO, A., and EADE, N. R. J. Physiol. **133**, 548 (1956).
41. FELDBERG, W., MINZ, B., and TSUDZIMURA, H. J. Physiol. **81**, 286 (1934).
42. BROWN, G. L. and GILLESPIE, J. S. J. Physiol. **138**, 81 (1957).
43. HOLZBAUER, M. and VOGT, M. J. Neurochem. **1**, 8 (1956).
44. DOMINO, E. E. and RECH, R. H. J. Pharmacol. Exptl. Therap. **121**, 171 (1957).
45. BURGEN, A. S. V., DICKENS, F., and ZATMAN, L. J. J. Physiol. **109**, 10 (1949).
46. NICKERSON, M. Pharmacol. Revs. **1**, 27 (1949).
47. EXLEY, K. A. Brit. J. Pharmacol. **12**, 297 (1957).
48. AHLQUIST, R. P. Am. J. Physiol. **153**, 586 (1948).
49. NICKERSON, M. and GOODMAN, L. S. J. Pharmacol. Exptl. Therap. **89**, 167 (1947).
50. POWELL, C. E. and SLATER, I. H. J. Pharmacol. Exptl. Therap. **122**, 480 (1958).
51. NICKERSON, M. Rev. can. biol. **14**, 275 (1955).
52. NICKERSON, M., HENRY, J. W., and NOMAGUCHI, G. M. J. Pharmacol. Exptl. Therap. **107**, 300 (1953).
53. NICKERSON, M. Nature, **178**, 697 (1956).
54. STEPHENSON, R. P. Brit. J. Pharmacol. **11**, 379 (1956).
55. FOLKOW, B. Acta Physiol. Scand. **25**, 49 (1952).
56. SCHAYER, R. W. and SMILEY, R. L. J. Biol. Chem. **202**, 425 (1953).
57. CORNE, S. J. and GRAHAM, J. D. P. J. Physiol. **135**, 339 (1957).
58. RITCHER, D. J. Physiol. **98**, 361 (1940).
59. CLARK, W. G., AKAWIE, R. I., POGRUND, R. S., and GEISSMAN, T. A. J. Pharmacol. Exptl. Therap. **101**, 6 (1951).
60. ARMSTRONG, M. D., MCMILLAN, A., and SHAW, K. N. F. Biochim. et Biophys. Acta, **25**, 422 (1957).
61. AXELROD, J. Science, **126**, 400 (1957).
62. BAIN, W. A., GAUNT, W. E., and SUFFOLK, S. F. J. Physiol. **91**, 233 (1937).
63. RAAB, W. and HUMPHREYS, R. J. J. Pharmacol. Exptl. Therap. **88**, 268 (1946).
64. NICKERSON, M., BERGHOUT, J., and HAMMERSTROM, R. N. Am. J. Physiol. **160**, 479 (1950).
65. DAWES, G. S. Brit. J. Pharmacol. **1**, 21 (1946).
66. ELLIOTT, T. R. J. Physiol. **32**, 401 (1905).

FORMATION, STORAGE, AND RELEASE OF ACETYLCHOLINE AT NERVE ENDINGS[1]

F. C. MacIntosh

Structure–Function Correlations

Three specific substances have been shown to be present at cholinergic synapses: (*a*) the transmitter itself, acetylcholine (ACh); (*b*) choline acetylase, the enzyme that makes ACh by transferring acetyl groups from coenzyme A to choline; and (*c*) acetylcholinesterase, the enzyme that destroys ACh. These three substances are not confined to the synaptic region: they are found in significant amounts along the whole length of every nerve trunk that contains cholinergic axons. In each case, however, the concentration is undoubtedly highest at the nerve endings. The function of each of these substances at the nerve terminals is plain enough because we know that ACh is formed, stored, released, and hydrolyzed there whenever impulses are transmitted. But why should they be present also in the axons, where they have not been shown to have any function? The most likely explanation is that the enzymes, choline acetylase and acetylcholinesterase, are synthesized in the nerve-cell body and are carried to the nerve endings by the axoplasmic current streaming down each fiber. At the endings they accumulate, like débris washed up on a weir. In support of this idea the following experiment may be cited. If you make a kind of artificial nerve ending by severing a nerve trunk that contains cholinergic fibers, and then a few days later examine the nerve above and below the cut, you will find that all three substances—ACh, choline acetylase, and acetylcholinesterase—have piled up at the end of the proximal stump and have disappeared from the degenerating part of the nerve and its endings (32, 15, 33). We are immediately reminded of the evidence for the transport of the neurohypophysial hormones along the nerve fibers running from the supraoptic nucleus to the pituitary, and we can suppose that cholinergic neurons are also in a way neurosecretory: with this main difference, that in the case of cholinergic neurons the material transported along the axon includes not only the active substance destined for release at the nerve ending, but also the enzymes that create and destroy it. Indeed the presence of the active material, ACh itself, is perhaps merely an indication of the fact that wherever an enzyme of synthesis exists in contact with its substrates, a certain amount of the finished product is bound to be made. Professor Nickerson will no doubt refer to the fact that adrenergic axons contain and can synthesize noradrenaline; and we may be sure that a variety of other neurohumors and specific enzymes, as yet unidentified, will eventually be discovered in sensory nerve trunks and in the grey and white matter of the central nervous system.

[1]Manuscript received October 20, 1958.
Contribution from the Department of Physiology, McGill University, Montreal, Que.

With this convenient explanation for the presence of ACh and its associated enzymes in the proximal parts of the neuron, we can concentrate our attention on the nerve endings, for it is only in the case of these minute organs that we have direct evidence for a rapid turnover of cellular acetylcholine during activity. Dr. Burgen has reminded us of the recent advances in our knowledge of neuronal ultrastructure and its biochemical correlates; and it should be worth while to try to match these new findings with what has already been learned by more conventional physiological and pharmacological methods. The latter sort of information has mostly been obtained in one of two ways. First, one can make records of electrical events in postsynaptic structures, and from them one can sometimes make valid deductions about presynaptic events: this is fortunate, for the presynaptic endings themselves are almost always too small for direct investigation with microelectrodes. Secondly, one can use bio-assay techniques to measure the ACh bound within the intact nerve endings, and also the ACh released from them into a perfusion fluid or a suspension medium. The electrical methods are essential when one wants to study events that take place within seconds or milliseconds; the bio-assay methods give reliable results when the endings are in a steady state of rest or activity. I will be speaking mostly about the results of bio-assay experiments, and some of the material I will present will be based on experiments by Dr. Richard Birks and myself with the perfused superior cervical ganglion of the cat. This preparation was first introduced for the study of acetylcholine metabolism by Brown and Feldberg (4) about 20 years ago. It is convenient for several reasons: first because it is easy to isolate its circulation and to control its activity, and secondly because it has a very high concentration of synapses, so that the dilution factors are favorable for bio-assay experiments. A further useful point is that the two ganglia in a cat have practically the same ACh content: thus by extracting the control ganglion one can find out how much ACh was in the other ganglion at the start of the experiment.

We can make a start by considering the probable location of the three substances we are dealing with. In a structure like a ganglion, as I have already pointed out, each substance disappears almost completely within a few days after the preganglionic fibers have been cut. Since the other cellular elements of the ganglion remain intact, we can conclude that our substances must have been located in the presynaptic elements: that is to say, the distal portions of the axons and their endings. By extracting and assaying the nerve trunk we can allow for the contribution made by the preterminal portions of the axons: this fraction is always small, so that in the intact ganglion most of the active material must be packed into the nerve endings. At this point we may recall that two of our three substances, ACh and cholinesterase, are incompatible and cannot exist together in solution, since the base is quickly destroyed by the enzyme. Presumably the ACh is separated from the cholinesterase by a membrane barrier. It is unlikely that ACh is held within the tissue by a chemical bond, since it can be extracted by mild procedures like simple grinding or treatment with distilled water or acetone in the cold.

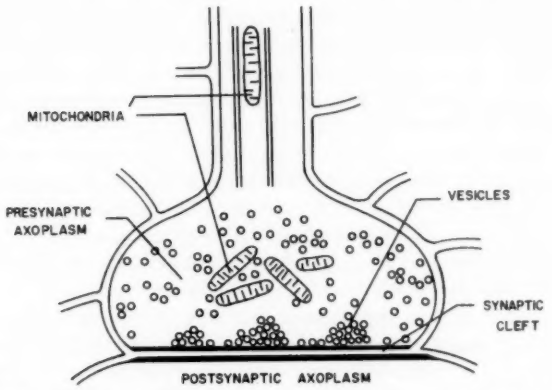


FIG. 1. Schematic representation of a synapse. The presynaptic ending is surrounded by the processes of glial or satellite cells.

Many types of axonal ending, cholinergic and otherwise, have now been examined in thin sections under the electron microscope. Though there is much variation in their pattern and size, they all exhibit certain common features. These are indicated diagrammatically in Fig. 1.

The ending is separated by a cleft about 200 Å broad from the postsynaptic neuron which it innervates, and by a scarcely wider gap from the glial or satellite cells that surround it: there is no other extracellular space in the immediate neighborhood of a synapse. The presynaptic axoplasm contains a number of mitochondria, suggestive of a brisk oxidative metabolism, and also a great number of smaller hollow spherical elements, the synaptic vesicles, which are the characteristic organelles of efferent nerve endings. These vesicles are unevenly distributed in the axoplasm and opposite the synaptic cleft they tend to be bunched against the presynaptic membrane, which here appears to be thickened. There are few if any such vesicles in the axon above the ending, or in the other cellular structures around the synapse; and the various anatomists (27, 30, 31) who first saw these small particles a few years ago all immediately and independently suggested that they represented the depots in which the synaptic transmitter was stored prior to its release.

In the case of at least one kind of cholinergic synapse we now have strong evidence that this suggestion is correct. Fatt and Katz (11), recording with intracellular microelectrodes from resting frog muscle fibers, regularly observed minute transient voltage fluctuations in the region of the motor end plate. They called them "miniature end-plate potentials" and were able to show conclusively that they were caused by the discontinuous leakage of ACh from the motor nerve ending: each of these minute events involved the discharge onto the muscle surface of a packet or "quantum" made up of a few hundred ACh molecules, an amount far too small to excite a propagated twitch response

of the muscle fiber. Further analysis by Castillo and Katz (5, 7) revealed that the full-sized "end-plate potential," which is the primary response evoked in a muscle fiber by a motor nerve impulse, is actually the sum of a large number of such minute effects, occurring almost simultaneously when the nerve ending is depolarized by the arrival of an impulse. Thus there must be in every motor nerve ending a great number of discrete small packets of preformed ACh. At rest these are released one at a time in random fashion, but during activity some hundreds of them are discharged in synchrony by every nerve impulse. The discovery of the synaptic vesicles provided a neat morphological correlate for these electrophysiological observations. It is hard to avoid the conclusion that each packet or quantum is in fact the amount contained in one vesicle, and that the release of ACh occurs when vesicles collide with or penetrate the presynaptic membrane and discharge their contents into the synaptic gap. On this assumption Dr. Birks made a rough estimate, shown in Table I, of the concentration of ACh within the vesicular fluid: the level approaches isotonicity, so ACh must be one of the most abundant cations in that fluid.

TABLE I

ACh in synaptic vesicles at cat's motor nerve endings

Assumptions

1. Each vesicle contains 1 quantum of ACh (Castillo and Katz (7))
2. Vesicle diameter is 400 Å (Palade (26))
3. Vesicle membrane is 50 Å thick
4. A single volley discharges 300 quanta in cat *tenuissimus* (Martin (25))
5. A single volley discharges 5×10^{-12} g ACh in cat *tibialis anticus* (Emmelin and MacIntosh (10))
6. Cat *tibialis anticus* contains 380 motor units (Denny-Brown (9))
7. White muscles of cat contain 165 fibers/motor unit (Clark (8))

Calculations

$$\text{ACh in 1 vesicle} = \frac{5}{300\times380\times165}\times10^{-12}\text{ g}$$
$$= 1.5\times10^{-21}\ M = 400\text{ molecules}$$
$$\text{Volume of 1 vesicle} = 1.3\times10^{-17}\text{ ml}$$
$$\text{Concentration of ACh in vesicular fluid} = \frac{1.5\times10^{-18}}{1.3\times10^{-17}}\ M = 0.11\ M$$

As to the location of the two enzymes, choline acetylase and acetylcholinesterase, we are on less secure ground. Hebb and Smallman (14) have shown that choline acetylase sediments with the mitochondrial fraction in sucrose homogenates of brain. In such preparations the enzyme does not exhibit its full activity, even when plenty of substrate is present in the suspending fluid: to get optimal ACh synthesis it is necessary to treat the suspension with ether, which apparently disorganizes some membrane barrier that prevents the access of the substrates to the enzyme. It may be, as Hebb and Smallman suggest, that the choline acetylase is in the mitochondria. Certainly it is in some sort of particle; but on the other hand it seems possible that the preparations of Hebb and Smallman contained other intracellular

particles, or even whole nerve endings.[2] Teleologically one might wish to have the synthesizing system located somewhere other than inside the vesicles, so that it would not be discharged and wasted whenever the transmitter was released; but to postulate this raises the difficult question of how the newly synthesized ACh could be loaded into the vesicles. If Hebb and Smallman are right in positing a mitochondrial location for choline acetylase, we should expect that some ACh would also be held temporarily in the mitochondria before being transferred to the vesicles. This would provide a convenient explanation for the presence of ACh in cholinergic axons, which contain mitochondria but no vesicles, and it might also explain the finding by Birks and myself (3) that some 10 or 15% of the ACh in a ganglion cannot be mobilized by prolonged preganglionic stimulation.

As to acetylcholinesterase, both histochemical and physiological evidence indicates that it has a dual location. Some of it appears to be attached to the outer surface of the presynaptic membrane, where its role is to destroy the released transmitter and so to limit the duration of its action. The rest of it is intracellular, and appears to be associated with some sort of membrane. Koelle (21), who has supplied most of the histochemical evidence, suggests that the cholinesterase within the neuronal cell body may be attached to the endoplasmic reticulum. This system, however, is absent from the nerve ending, unless the vesicles can be taken to represent it. The intracellular cholinesterase of the nerve ending may rather be on the outer face of the vesicles or on the inner face of the presynaptic membrane. The neuromuscular junction is an anomalous synapse, in that a large part of the cholinesterase is located on the postsynaptic face of the junction and survives the denervation of the muscle.

Acetylcholine Turnover at Nerve Endings

I should like now to describe some of our experiments with perfused ganglia, because they illustrate rather well some of the processes that occur at cholinergic endings during rest and activity.

A resting superior cervical ganglion from a cat, with its normal blood supply, contains about 0.25 μg of ACh, and this content is not much changed during synaptic activity. The same is true of a perfused ganglion, provided normal heparinized plasma is used as the perfusion fluid. But although the ACh content stays the same, ACh turnover is occurring: slowly during rest, and briskly during activity. This becomes evident when an anticholinesterase drug is added to the perfusion fluid. We might look first at what happens in the resting ganglion.

Fatt and Katz's (11) discovery of the spontaneous quantal discharge implies that there must be a continuous trickle of ACh release from resting nerve endings. By pushing our assay methods to their limit we were able to detect such a resting discharge in ganglia perfused with an eserinized fluid. It amounted to about 0.3 mμg/min, which means that it would take a resting

[2]Hebb and Whittaker (16) have recently reported evidence that choline acetylase and ACh of the mitochondrial fraction are located in some kind of particle other than the mitochondria.

ganglion from 12 to 24 hours to turn over the whole of its contained ACh. Besides this ACh that escapes into the perfusion fluid, there is also some further ACh turnover which is confined to the interior of the nerve endings. This can be seen when the ACh content of a series of ganglia is measured after an anticholinesterase drug has been given (Fig. 2); in this case TEPP, but eserine has the same effect. The content rises steadily over an hour or two and eventually reaches more than double the initial value. This "surplus ACh" is only formed when the perfusion medium is one that is favorable for ACh synthesis, and it disappears quickly if the cholinesterase is reactivated. It is intracellular, since it does not escape into the perfusate or stimulate the ganglion cells; but it must be in a different location from the regular ACh of the nerve endings, for unlike the latter it is not released by nerve impulses. "Surplus ACh" has no physiological significance, but its formation shows that ACh is continuously being made and destroyed at a slow rate in resting nerve endings. It also suggests that the newly formed ACh is not loaded into the vesicular depots with 100% efficiency: some spills over into another compartment, presumably the presynaptic axoplasm.

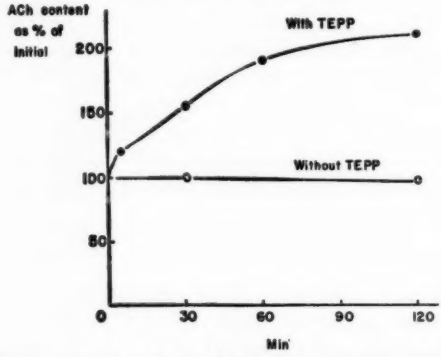


FIG. 2. ACh content of unstimulated ganglia (expressed as percentage of ACh in control ganglia excised at start of each experiment). Above: data from experiments in which TEPP was given by vein (1 mg/kg every 5 minutes); the preganglionic trunk had been sectioned and the animals were maintained on artificial respiration and protected with atropine and gallamine. Below: data from experiments on animals similarly treated except for the administration of TEPP.

We can turn now to the events in active nerve endings. It is convenient to start with a repetition of Brown and Feldberg's (4) experiment, in which they first undertook to measure ACh metabolism at synaptic regions. They perfused a ganglion with eserinized Locke's solution and stimulated the preganglionic trunk for an hour or so, and they got results very much like those shown in the middle curve of Fig. 3*a*, which shows the mean values from five experiments by Birks and myself. The ACh output tapers off more or less exponentially and eventually reaches a comparatively low level, at which it remains steady. Here the stimulation frequency was 20/second, but one gets almost identical figures if one excites the preganglionic nerve at higher or lower

frequencies (28), or if instead of exciting the nerve one depolarizes the nerve endings by perfusing with Locke enriched with K^+. Brown and Feldberg interpreted their results by suggesting—quite rightly, as we shall see—that during the falling part of the curve the ganglion's ACh depots were being depleted faster than they could be replenished, while in the flat part of the curve release and synthesis were in balance.

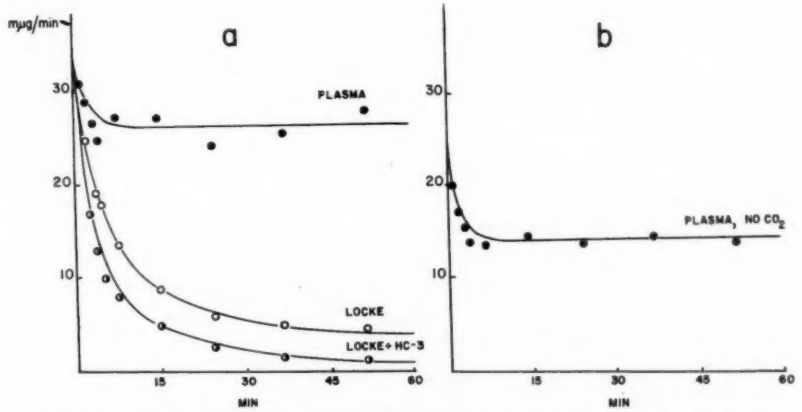


FIG. 3(*a*). ACh output from eserinized superior cervical ganglia during 1 hour's preganglionic stimulation at 20/second (each curve gives the mean values for five experiments). Perfusion fluids from above downward: heparinized cat plasma equilibrated with 5% CO_2 in O_2, oxygenated Locke's solution, oxygenated Locke's solution containing HC-3 (2×10^{-5} *M*).

(*b*). ACh output from ganglia similarly stimulated, during perfusion with eserinized plasma equilibrated with CO_2-free oxygen (mean of five experiments).

Locke's solution is hardly a complete physiological medium, and Fig. 3*a* also shows this experiment repeated with two other perfusion fluids, again with five trials for each fluid. At the top are the results with eserinized plasma. With this fluid, the output starts at the same level as with Locke, falls off a little in the first 5 minutes, but then stays practically steady at a high level for as long as stimulation goes on. Exactly opposite results are obtained, as in the lowest curve, when the perfusion fluid contains the hemicholinium base HC-3, about which I shall have more to say presently. When this drug is present it does not matter much whether the perfusion fluid is Locke or plasma; in either case the ACh output rate falls off more steeply than before and eventually reaches negligibly low levels. One suspects immediately that plasma supports ACh synthesis better than Locke does and enables synthesis to keep pace with output; and one also suspects that HC-3 stops ACh synthesis, so that ACh release stops when the preformed stock has been exhausted.

These suspicions are proved to be correct when one compares the acetylcholine output of the ganglia with the change that has occurred in their

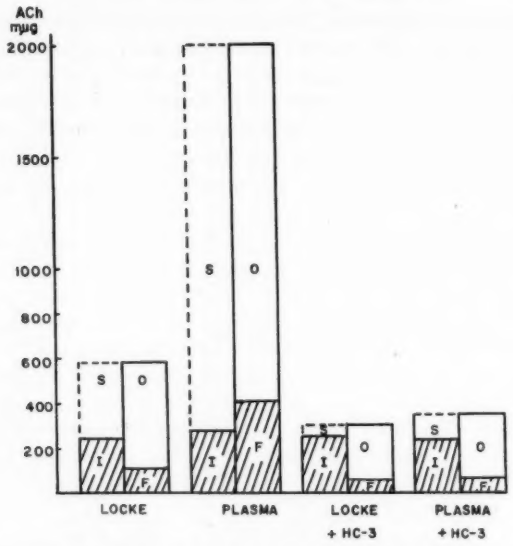


FIG. 4. ACh metabolism of stimulated ganglia: effect of changing the composition of the perfusion fluid. I, initial ACh content of ganglion (taken as equal to ACh in control ganglion). F, final ACh content after preganglionic stimulation for 60 minutes at 20/second. O, total amount of ACh discharged into effluent during stimulation. S (= F+O−I), calculated amount of ACh synthesized during stimulation.

acetylcholine during the stimulation. Figure 4 gives the balance sheet for each kind of experiment: the initial ACh content as determined by assaying the control ganglion, the final ACh content, the integrated ACh output during 60 minutes of stimulation, and (to complete the balance sheet) the calculated amount of ACh that must have been synthesized. The Locke-perfused ganglia during 1 hour's activity have synthesized a good deal of ACh, but they have lost about half their initial stock, and it could be shown that some of their remaining content was ACh of the "surplus" unreleasable kind. The depot ACh, indeed, was reduced in parallel with the reduction of ACh output: a fact that the earlier workers for technical reasons unfortunately failed to observe. The plasma-perfused ganglia have kept their ACh stock intact in spite of having released a great deal more of the ester, and they have also piled up some surplus ACh that they cannot release. The ganglia poisoned with HC-3 have hardly synthesized any, and their ACh is down to 15–20% of its starting level, most of the remainder being in the unreleasable fraction. Thus plasma contains some factor that promotes synthesis, so effectively indeed that it looks as if junctional fatigue due to diminished output of the transmitter can never occur under physiological conditions. HC-3 on the other hand is an inhibitor of synthesis. But since the *initial* rate of output of ACh was the same with all the perfusion fluids, none of these factors, apparently, affected ACh release.

It is possible, however, to have defective release of ACh as well as defective synthesis. The plasma used in the last experiments was equilibrated with a CO_2–O_2 mixture to bring its pH to 7.4. When we leave the CO_2 out and gas the plasma with O_2 alone, the pH rises above 8 and we get a different result (Fig. 3*b*). The output rate is lower from start to finish than in the first series of plasma experiments, but this is not because there is any shortage of ACh in the nerve endings: indeed the final content is significantly higher than when CO_2 is present. It is because each preganglionic volley sets free a smaller proportion of the ACh in the depots. The effect of CO_2 on ACh release is probably due to increased ionization of plasma calcium. Harvey and I (13) showed many years ago that Ca ions are necessary for ACh release; and more recently Hutter and Kostial (18) in perfusion experiments, and Castillo and Katz (5) by electrophysiological analysis, have shown that the amount of ACh released varies with the Ca ion concentration over a wide range. They have also shown that Mg ions have exactly the opposite effect: Mg interferes with ACh release, and antagonizes the action of Ca.

By varying the composition of the perfusion fluid in other ways, and determining the effect on ACh content and ACh output, one should be able to identify the plasma factors that are important for synthesis and release of ACh under physiological conditions. In perfusion experiments lasting an hour or so the only important factors are the ones shown in Table II.

TABLE II

Factors affecting synthesis and release of depot ACh

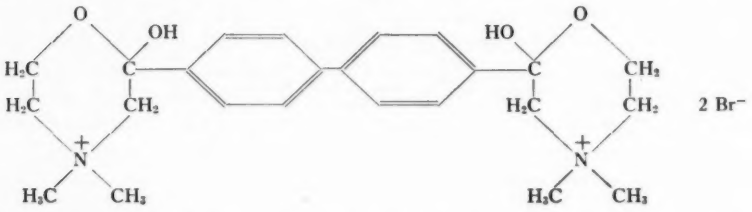
Synthesis	Release
Necessary	*Necessary*
1. Glucose	1. Depolarization of nerve membrane (e.g. by nerve impulses, excess of K, anoxia, etc.)
2. Choline	2. Cations
3. Na ions	*Necessary for optimal release*
	1. CO_2
	2. Unidentified plasma factor (dialyzable and $CHCl_3$-soluble)
Inhibitory	*Inhibitory*
HC-3 and other hemicholiniums (strong)	Mg^{++}
Some other quaternaries (weak)	Botulinus toxin
Eserine (weak)	

For efficient *synthesis* of ACh, the Locke medium has to be supplemented with only one substance—choline: there is enough choline in normal plasma to support optimal synthesis, however heavy the traffic of nerve impulses may be. For efficient *release* of ACh, a dialyzate of plasma is as good as plasma itself: besides Ca^{++} and CO_2, it contains at least one substance, apparently a fat-soluble one of low molecular weight, which assists release: we have not as yet tried to identify it. I might add that normal human plasma is indistinguishable from cat plasma in its effects on the ganglion, and so is plasma from

patients with severe myasthenia gravis. Of the constituents of Locke's solution, Na^+ appears to be necessary for ACh synthesis, but not for ACh release provided excess of K^+ or some other factor is present to depolarize the nerve endings, in lieu of the nerve impulse which cannot travel in the absence of Na^+ (19); and glucose (or some related fuel) and oxygen must be supplied for optimal synthesis, as has long been known (24, 20).

Choline Transport and Its Inhibition

I should like to spend most of the rest of my time discussing the drug HC-3, which has proved to have an interesting and unexpected mode of action. It belongs to a group of bases synthesized a few years ago by Long and Schueler (22), which Schueler (34) has named the hemicholiniums. Schueler (34) proved that the characteristic toxicity of these compounds is associated with their unusual chemical structure. This is exemplified in the formula of HC-3.



The business end of the molecule—or two business ends in this case—is a choline moiety cyclized through hemiacetal formation into a six-membered ring. Schueler further showed that hemicholinium poisoning had the characteristics shown in Table III.

TABLE III

Characteristics of hemicholinium intoxication (34)

1. Death is due to respiratory failure
2. Signs of poisoning appear after a latent period whose length is unrelated to the dose of the drug
3. Artificially respired animals withstand large doses of the drug
4. Pupils become dilated and fixed
5. Eserine and choline are effective antidotes

It was these observations of Schueler's that suggested to us that HC-3 might be a specific inhibitor of ACh synthesis, and indeed this idea had already occurred to Schueler. We were both misled for a time, however, by finding that the compound had only a very weak inhibitory action on choline acetylase in solution: our own tests were done on extracts of acetone-dried brain incubated with choline and acetyl coenzyme A. We also eliminated the possibility that the drug might act by inhibiting the acetylation of coenzyme A. It did, however, act powerfully, as we have already seen, on acetylcholine synthesis in the ganglion, and almost as strongly on ACh

synthesis in minced brain: furthermore, it was clear that it was competing in some way with choline, for all its effects could be antagonized by raising the choline concentration of the medium. We therefore suggested in our first note (23) about the drug that it might act as an inhibitor of choline transport, i.e. by preventing the access of extracellular choline to the intracellular sites of acetylation.

There are now three pieces of evidence to support this hypothesis. *First* there is the finding (3) that though choline behaves like an inert non-penetrating ion with respect to nerve axons, a ganglion can take it up with great efficiency: a plasma-perfused ganglion indeed can capture and acetylate more than 20% of the exogenous choline supplied to it during the few seconds required for the plasma to pass through the ganglionic capillaries. It is hard to see how this could happen unless the nerve endings were equipped with some special kind of portal for the ingress of choline ions. *Secondly*, Gardiner (12) has recently reported, from Paton's laboratory, that the inhibitory action of HC-3 on brain ACh synthesis is much less in homogenates than it is in minced tissue, and disappears when the homogenates are treated with ether. This is what one would expect if the harsher treatment of the tissue had disrupted membrane barriers to the diffusion of choline. *Thirdly*, there is a series of experiments by my colleague Dr. Richard Birks (2) on the kidney of the hen. It is known that this organ, and probably the mammalian kidney too, can actively excrete choline (29) and various other organic bases by a tubular mechanism quite distinct from the one involved in the excretion of substances like PAH and phenol red: there is no reason I know of to think that this renal transport of choline involves its acetylation, but it must certainly involve a fairly specific carrier mechanism. Figure 5 shows that HC-3 interferes with that mechanism. Phenol red was used as a control substance, and it was delivered along with choline by continuous infusion into the renal portal vein supplying the tubules of one kidney. Both substances were efficiently excreted by that kidney and only a little reached the opposite kidney. When HC-3 was added to the infusion fluid the excretion of phenol red was unaffected, while the excretion of choline was inhibited as long as HC-3 was present.

I think all this is pretty good evidence for our hypothesis, and that it is not too dangerous to conclude that cholinergic transmitter systems must include a fourth specific component in addition to the three we have been discussing: a choline carrier located in some membrane at the synapse. More work is needed to show whether the transport is active or passive, and also to localize the carrier sites more accurately. These sites might be in the presynaptic membrane itself, or in some kind of particle within the nerve ending, or even in the glial or satellite cells which invest the nerve endings and form most of their fluid environment.

HC-3 seems to be a relatively "clean" pharmacological agent in that most of its effects can be explained by this one property. So far as our rather limited exploration goes, it will block transmission in any peripheral cholinergic

pathway, but only if that pathway has been active long enough for the depot ACh at the nerve endings to be exhausted. When the pathway is infrequently excited, no block develops at all. One illustration will have to suffice (Fig. 6). This is the response of a cat's nictitating membrane to 15-minute nerve stimulation at 20/second; the ganglion retained its blood supply, and the animal was artificially respired so that it was not killed when HC-3 was injected. Before the drug the response to preganglionic stimulation was well maintained; after the drug, the initial response was normal but it soon began to fail, but the effector organ could still maintain a good response to postganglionic stimulation. The story with other cholinergic effectors is much

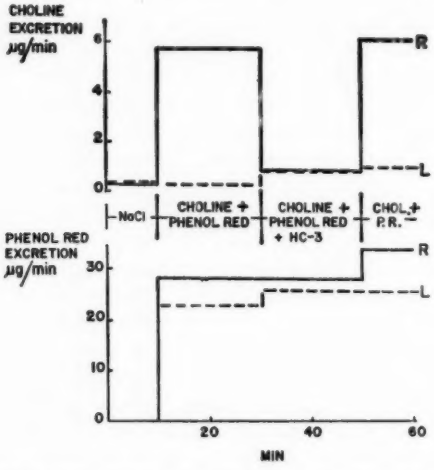


FIG. 5. Excretion of choline and phenol red during continuous infusion of these substances into right renal portal vein of anaesthetized chicken. Continuous line, output from right kidney; dashed line, output from left kidney. For details, see (2).

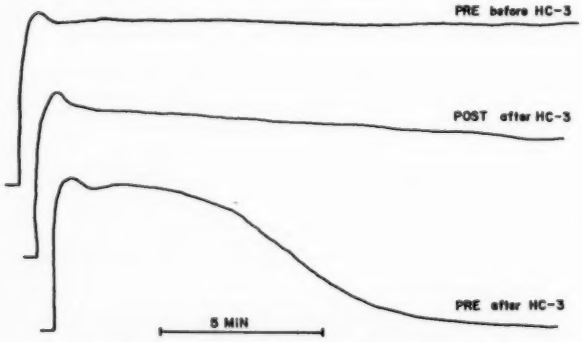


FIG. 6. Responses of cat nictitating membrane to continuous stimulation of pre- or post-ganglionic cervical sympathetic trunk, before and after intravenous administration of HC-3 (1 mg/kg), showing the drug's characteristic delayed synaptic-blocking action.

the same. The drug has little direct ganglion-blocking, anticholinesterase, or atropine-like activity; in large doses, however, it has some immediate neuromuscular-blocking activity (34). The specific block in repetitively excited pathways can always be relieved at least in part by rest, or by lowering of the stimulation frequency, or by the injection of choline.

Triggering of Acetylcholine Release and Acetylcholine Synthesis

I am conscious that I have not begun to answer the two most important questions that might be asked about presynaptic mechanisms. The first one is: "Why does depolarization of the nerve ending bring about the release of transmitter from the depots?" The second one is: "Why does the release of the transmitter accelerate its synthesis, so that the depots are kept stocked during activity?" Questions of this sort, about how cellular processes are coupled together, are generally hard to answer, and these two are no exception.

As to the mechanism of release, Birks and I (1) and Hodgkin and Keynes (17) have both suggested that this involves the passage of Ca ions into the presynaptic axoplasm. Hodgkin and Keynes, who have studied the movement of Ca into and out of squid nerve fibers, find that normally there is very little Ca in the axoplasm, but that during activity there is a sharp rise in Ca influx, while during rest the Ca that has entered is slowly pumped out. If nerve endings handle Ca in the same way as axons do, one might guess that when an impulse arrives at the nerve ending a few Ca ions are allowed to leak in, and that this Ca alters the properties of the vesicles or the axoplasm just inside the membrane in such a way as to induce the vesicles to spill their contents into the synaptic gap.

This line of speculation may perhaps be extended a little further. It can be shown in several ways (3) that the depot ACh at the nerve endings, before being set free, is first somehow transformed so that its chance of being released by subsequent nerve impulses is increased about tenfold. In a ganglion that has been rested, about a fifth of the depot ACh is in this labile or readily releasable state. Probably this fraction represents the ACh in those vesicles that are close to or stuck to the presynaptic membrane. Now it is well known that at many kinds of junction an important phenomenon usually called "post-tetanic potentiation" can be demonstrated: the effect of a nerve impulse arriving at a synapse is greatly enhanced if a series of impulses have recently arrived over the same pathway. There is good reason to believe that post-tetanic potentiation depends on the release of a greater amount of transmitter per nerve impulse: presumably on the discharge of a greater number of quanta. It is significant that at the motor end plate during the period of potentiation there is a striking increase in the frequency of the spontaneously occurring miniature potentials (6). One might hazard the guess that for some time after a tetanus there is an increased amount of Ca within the motor terminals, and as a result a greater proportion of the vesicles are readily mobilized. Perhaps it should be added that the phenomenon here considered has no clear relationship to the longer-lasting changes in synaptic

conductance associated with use and disuse, conditioning, and memory: it is not yet possible to say how far these changes are presynaptic and how far postsynaptic in origin. As to the second question—"how is synthesis triggered by release?"—there are some hints that the inflow of Na ions which also accompanies activity may be the most important factor here. Perhaps Na within the ending stimulates vesicle formation, and by removing the product, ACh, from the vicinity of the synthesizing enzyme allows synthesis to continue as fast as the supply of substrate will allow. But as yet such hypotheses as these have slender experimental support, and a lot of further work will be required to establish them.

References

1. BIRKS, R. I. and MACINTOSH, F. C. Brit. Med. Bull. **13**, 157 (1957).
2. BIRKS, R. I. and MACINTOSH, F. C. Neurology, **8**, Suppl. 1, 90 (1958).
3. BIRKS, R. I. and MACINTOSH, F. C. To be published.
4. BROWN, G. L. and FELDBERG, W. J. Physiol. **88**, 265 (1936).
5. DEL CASTILLO, J. and KATZ, B. J. Physiol. **124**, 560 (1954).
6. DEL CASTILLO, J. and KATZ, B. J. Physiol. **124**, 574 (1954).
7. DEL CASTILLO, J. and KATZ, B. Progr. Biophys. **6**, 121 (1956).
8. CLARK, D. A. J. Physiol. **70**, 18P (1930).
9. DENNY-BROWN, D. Selected writings of Sir Charles Sherrington. Hamish Hamilton, London. 1939. p. 498.
10. EMMELIN, N. and MACINTOSH, F. C. J. Physiol. **131**, 477 (1956).
11. FATT, P. and KATZ, B. J. Physiol. **117**, 109 (1952).
12. GARDINER, J. E. J. Physiol. **138**, 13P (1957).
13. HARVEY, A. M. and MACINTOSH, F. C. J. Physiol. **97**, 408 (1940).
14. HEBB, C. O. and SMALLMAN, B. N. J. Physiol. **134**, 385 (1956).
15. HEBB, C. O. and WAITES, G. M. H. J. Physiol. **132**, 667 (1956).
16. HEBB, C. O. and WHITTAKER, V. P. J. Physiol. **142**, 187 (1956).
17. HODGKIN, A. L. and KEYNES, R. D. J. Physiol. **138**, 253 (1957).
18. HUTTER, O. F. and KOSTIAL, K. J. Physiol. **124**, 234 (1954).
19. HUTTER, O. F. and KOSTIAL, K. J. Physiol. **129**, 159 (1955).
20. KAHLSON, G. and MACINTOSH, F. C. J. Physiol. **96**, 277 (1939).
21. KOELLE, G. B. and STEINER, E. C. J. Pharmacol. Exptl. Therap. **118**, 420 (1956).
22. LONG, J. P. and SCHUELER, F. W. J. Am. Pharm. Assoc. (Sci. Ed.) **43**, 79 (1954).
23. MACINTOSH, F. C., BIRKS, R. I., and SASTRY, P. B. Nature, **178**, 1181 (1956).
24. MANN, P. J. G., TENENBAUM, M., and QUASTEL, J. H. Biochem. J. **32**, 243 (1938).
25. MARTIN, A. R. J. Physiol. **130**, 114 (1955).
26. PALADE, G. E. J. Biophys. Biochem. Cytol. **2**, Suppl. 85, (1956).
27. PALADE, G. E. and PALAY, S. L. Anat. Record, **118**, 335 (1954).
28. PERRY, W. L. M. J. Physiol. **119**, 439 (1953).
29. RENNICK, B. R. J. Pharmacol. Exptl. Therap. **122**, 449 (1958).
30. DE ROBERTIS, E. D. P. and BENNETT, H. S. J. Biophys. Biochem. Cytol. **1**, 47 (1935).
31. ROBERTSON, J. D. J. Biophys. Biochem. Cytol. **2**, 381 (1956).
32. SASTRY, P. B. Ph.D. thesis. McGill University, Montreal. 1956.
33. SAWYER, C. H. Am. J. Physiol. **146**, 246 (1956).
34. SCHUELER, F. W. J. Pharmacol. Exptl. Therap. **115**, 127 (1955).

SYMPOSIUM ON NEUROSECRETION

DISCUSSION: *Edited by* A. S. V. Burgen

Dr. D. de Wied.—In connection with Dr. Noble's and Dr. Saffran's presentations, I would like to tell you of some experiments recently carried out in Dr. Mirsky's department in Pittsburg. High doses of prednisolone were used to block the release of ACTH following stressful stimuli, such as pain and histamine, and nicotine injections. As ADH has been postulated to be responsible for ACTH release, the effect of the same doses of prednisolone on ADH release were investigated. It appeared that the release of ADH under the same conditions was not inhibited by prednisolone. Thus in the presence of ADH no ACTH release is observed. Hence ADH is not responsible for ACTH secretion because it is known that pitressin is capable of releasing ACTH in prednisolone-treated rats. Blood withdrawal provokes a tremendous rise in ADH activity in jugular vein blood of rats. Contrary to our previous findings, prednisolone partly prevented the release of ADH induced by blood withdrawal. In regard to the afore-mentioned, I would like to ask Dr. Noble the following questions:

First, is there a difference between the site of action of neurogenic stimuli and the stimulus evoked by blood withdrawal? Secondly, is it possible that ADH is secreted from the hypothalamic nuclei direct into the circulation? Thirdly, is it possible that corticoids which block the anterior lobe also block the posterior lobe of the pituitary gland? Further, I would like to ask Dr. Saffran if circulating ADH differs from purified vasopressin, and if so, could this account for the fact that vasopressin releases ACTH and that circulating ADH apparently fails to do so?

Dr. Noble.—I would like to thank Dr. de Wied for his interesting comments. With respect to the question of whether ADH is secreted directly, I think that in various histological preparations the grouping of the neurosecretory elements around the small blood vessels would certainly be very suggestive that the ADH may be a direct secretion even around the hypothalamic and supraoptic nuclei. Regarding the second question of whether the corticoids block posterior lobe function, at the moment I cannot recall any direct evidence for that. Perhaps the experiment Dr. Saffran quoted, where you hydrate an animal, suppress the release of ADH, and yet have a stimulation of ACTH, might be interpreted in that way. I cannot think of any direct experiments where there has been a blocking of posterior lobe hormones by the administration of corticoids. Dr. Fortier has postulated a dual control of the anterior pituitary hormones and he envisions that, since even the isolated gland will respond, you have a humoral type of control as well as a control through a nervous reflex through the portal system, and I think the removal of blood might come under the category of a direct effect rather than through the portal system.

Dr. Saffran.—Perhaps the answer to Dr. de Wied's question about the difference between isolated or synthetic vasopressin and that circulating in the blood may depend upon circumstances, and I touched upon it in the course of the talk. In cases where osmotic changes provoke the release of antidiuretic hormone, the chances are that the hormone is identical with the material that has been isolated and synthetized. When that particular hormone is kept within the pituitary by a load of water and then a stress is put onto the animal, the corticotropin-releasing factor may enter into the circulation and perhaps under conditions of stress, all by itself, without the water load, the increase in antidiuretic activity may be a subproperty of the corticotropin-releasing factor. To my knowledge there is no definite evidence for the identity of the circulating antidiuretic hormone.

Dr. Kalow.—I wonder whether it is possible to speculate as to whether the effect of CO_2 on release of acetylcholine may be of functional importance in the respiratory center or the carotid body?

Dr. Burgen.—This is a very interesting question, but as Dr. Kalow has indicated any suggestion that the respiratory center or carotid body are influenced by CO_2 tension through the intermediary control of acetylcholine release is highly speculative since we do not know whether this effect of CO_2 is common to other cholinergic synapses nor whether acetylcholine release is important at these two sites.

Dr. Beveridge.—I have always been very much interested in the dramatic sequence of events one sees when an animal has been given a diet low in magnesium, and I wonder if you would care to attempt to explain this on the basis of the presentations that have been given this afternoon.

Dr. Burgen.—I think it is a little dangerous to suppose that all the effects of magnesium deficiency can be tied down to this action on presynaptic release. There is no doubt that magnesium has other actions on effector organs and on neurones. But the idea of a presynaptic action would fit in with the well-known observation that an animal narcotized by the injection of magnesium can be awakened by the subsequent injection of calcium. It is only in the case of their action on presynaptic endings that these two ions, Ca and Mg, show a clear antagonism.

Dr. Rochefort.—I would like to mention that we have obtained evidence in our laboratory, and it has also been obtained in France, for the dual mechanism of ACTH release, that is that neurotropic stimuli will affect primarily the pituitary through the nervous system whereas systemic stimuli affect only the anterior pituitary, and also I would like to say that synthetic vasopressin, in vivo, has not been active in releasing ACTH from the pituitary. I would also like to ask Dr. Saffran if he would care to comment on the mechanism of CRF release, is it from the posterior lobe or from the hypothalamus?

Dr. Saffran.—On going back to your first statement, I think you implied that neurotropic stresses such as sound and restraint act by releasing ACTH from the neurohypophysis, whereas the systemic stresses, administration of drugs, changes in temperature, and so on, act on the ACTH in the adenohypophysis The major stores of corticotropin-releasing factor as far as we can tell are held in the neurohypophysis, at least it is easier to extract the material from the neurohypophysis than from the hypothalamus. This is also true, of course, in the case of vasopressin and oxytoxin. Arguing only by analogy perhaps the picture with the corticotropin-releasing factor is the same as that with the other hormones, they may be manufactured in the hypothalamus and stored and released from the neurohypophysis. The neurohypophysis is a rather large area comparatively speaking. It stretches from the pars tuberalis down to the other end of the posterior lobe, and in the pars tuberalis it is very difficult to say whether one should consider this to be hypothalamus or neurohypophysis, so that the release may be affected in the *pars tuberalis*. On the other hand, there are good bits of evidence that the *pars tuberalis*, the main part of the posterior lobe, and the main part of the anterior lobe are connected by blood vessels. These blood vessels have been seen in man as well as in the dog and rat, and there is a possible pathway for the transmission of material from the main body of the posterior lobe to the anterior lobe.

Dr. Burgen.—I should like to ask a question in reply to Dr. Rochefort, and that is what he means by neurotropic stimulus.

Dr. Rochefort.—Well, that is Dr. Fortier's division of stressful stimuli, those that apparently will affect the animal only by its nervous system, such as sound, or an intense light, or fright, or immobilization, whereas what he calls a systemic stimulus affects the animal through both its nervous system and the bloodstream, that is, for example, a burn, breakage of a limb, or a bacterial toxic infection. He divides the stresses into these two categories, and it has been shown that the neurotropic stimuli affect first of all the posterior lobe of the ACTH stores, and secondarily the anterior stores when the posterior lobe is empty, whereas systemic stimuli will affect only the anterior pituitary ACTH stores.

Dr. Burgen.—I am afraid I still do not see why breakage of a limb is not neurotropic. Surely you are stimulating the nervous system just as much by that as you are by intense sound.

Dr. Rochefort.—Yes; well, he calls a systemic stimulus that which affects the animal both by metabolic changes which may be carried by the bloodstream and through the nervous system, whereas the neurotropic one will not touch the animal, as it were; it will be simply through the nervous system, such as sight, or through hearing or immobilization.

Dr. Pierce.—Mr. Chairman, I wonder if any of the participants might be prepared to suggest a mechanism by which the activation of nerves to the posterior pituitary can result in a release of antidiuretic hormone. I may have missed a consideration of the subject, but I feel it is getting overlooked in the present presentation.

Dr. Noble.—I presume that by that question you mean what is the actual mechanism that releases the neurosecretory material so that it goes into the smaller blood vessels? I think that is probably a matter of pure speculation, but from the work of Verney on release of antidiuretic material in response to osmotic changes, one perhaps might think that the osmoreceptors are intimately concerned with the nerve endings, and perhaps with the alteration in permeability the material is released into the bloodstream, which is not really saying very much, but perhaps it is an osmotic effect at the terminal end of the nerve fibril, which is in very close proximity to the small blood vessels. I would stand for other suggestions.

Dr. Burgen.—If I may add to that, Dr. Noble, it must be remembered that electrical stimulation of the supraoptic nucleus will lead to liberation of the antidiuretic hormone, and I presume that there is no osmotic change under those circumstances. I think perhaps what Dr. Pierce is trying to get at is whether there is evidence for a transmitter action, let us say, in the supraoptic region, and there is some. Pickford has found that injecting an anticholinesterase locally into the supraoptic nucleus will produce changes in antidiuretic hormone release, suggesting that either the neurones containing the hormone are cholinergic themselves, or that some neurone playing on these is of the cholinergic type. I do not think it is settled in any way whether these effects are strictly on the neurones or not.

Dr. Saffran.—In our earliest work on the release of ACTH from the anterior lobe, instead of adding extracts of neurohypophysis or hypothalamus, we added pieces of neurohypophysis tissue, or bits of hypothalamus, and found that these pieces of tissue did not do anything to the release of ACTH from the adenohypophysis until either adrenaline or noradrenaline were added to the tissue. The combination of neurohypophysis and noradrenaline or hypothalamus and noradrenaline stimulated the release of ACTH, so that at least in the case of the corticotropin-releasing factor, there may be an adrenergic effect just prior to the release of the factor.

COMMONWEALTH BUREAU OF ANIMAL NUTRITION

ROWETT RESEARCH INSTITUTE, BUCKSBURN, ABERDEENSHIRE, SCOTLAND

Publications and Services

NUTRITION ABSTRACTS AND REVIEWS

World literature on the nutrition of man and animals is abstracted in sections embracing methods of analysis and experimental techniques; composition, digestion, and utilization of food and systems of feeding; growth, reproduction, and all physiological processes concerned with the use of food; the relation of diet to health and disease; the feeding of livestock.

Each number contains also a Review Article dealing with some subject of current practical importance.

Published quarterly. Price 126*s*. per volume. The current volume is Vol. 29.

TECHNICAL REPORTS

From time to time the Bureau prepares Technical Communications on subjects of practical importance. A list of these may be had on request.

CONSULTATION SERVICE

The Bureau is prepared, within the limits of its information, to answer enquiries from workers in laboratory research, field investigations, or nutritional planning, who do not themselves have the necessary access to literature.

Canadian Journal of Biochemistry and Physiology

NOTES TO CONTRIBUTORS

MANUSCRIPTS

General.—Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables, and captions for the figures, should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered. Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Greek letters or unusual signs should be written plainly and explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

Abstract.—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

References.—These should be designated in the text by a key number and listed at the end of the paper, with the number, in the order in which they are cited. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given, and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts*. All citations should be checked with the original articles.

Tables.—Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should not be used. Numerous small tables should be avoided.

ILLUSTRATIONS

General.—All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations.

Line drawings.—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines must be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots must be solid black circles large enough to be retained when reduced. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm high when the figure is reduced to a suitable size. Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. Wherever possible two or more drawings should be grouped to reduce the number of cuts required. In such groups of drawings, or in large drawings, full use of the space available should be made; the ratio of height to width should conform to that of a journal page (4½ × 7½ in.), but allowance must be made for the captions. **The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.**

Photographs.—Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard, with no space between those arranged in groups. In mounting, full use of the space available should be made. **Photographs are to be submitted in duplicate;** the duplicate set of grouped photographs should be unmounted.

REPRINTS

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased at the time of publication. Charges are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. Prices and instructions for ordering reprints are sent out with the galley proof.

Contents

Page

Fred J. McIlreath, Jr.—Some evidence concerning the mode of binding of histamine 151

R. E. Haist, Rebeka Moscarello, T. L. Friedlich, and J. R. Hamilton—The influence of environmental temperature and humidity on shock produced by a clamping procedure 165

Florent Depocas—Turnover of plasma glucose in anesthetized warm- and cold-acclimated rats exposed to cold 175

F. Sauer, D. H. Laughland, and W. M. Davidson—Silica metabolism in guinea pigs 183

C. D. Nelson and D. C. Mortimer—The preparation of sucrose-C^{14} with high specific activity and high tracer yield using detached sugar beet leaves . . 193

J. N. Baxter, A. S. Perlin, and F. J. Simpson—Preparation and assay of D-erythrose 4-phosphate 199

H. Schachter, S. Sidlofsky, D. G. Baker, J. R. Hamilton, and R. E. Haist—The effect of previous exposure to cold on shock secondary to limb ischaemia 211

H. M. Czajkowska Robinson and J. C. Rathbun—The determination of calcium and magnesium in blood using one indicator 225

Guy Nadeau and Louis-Philippe Joly—Étude de la réaction de l'adrénaline avec l'éthylènediamine 231

S. W. Levy—The activation and determination of plasmin in the rat 245

E. H. Colhoun—Acetylcholine in *Periplaneta americana* L. III. Acetylcholine in roaches treated with tetraethyl pyrophosphate and 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane 259

Frank A. Sréter—The effect of systematic training on plasma electrolytes, haematocrit value, and blood sugar in thoroughbred race horses 273

Florent Depocas. With the technical assistance of V. Secours—The pool size, turnover rate, and oxidation rate of body glucose in anesthetized warm- and cold-acclimated rats exposed to a warm environment 285

J. F. Scaife and D. H. Campbell—The destruction of O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate by liver microsomes 297

SYMPOSIUM ON NEUROSECRETION

A. S. V. Burgen—Introduction 307

R. L. Noble—Neurohypophyseal secretion 309

Murray Saffran—Activation of ACTH release by neurohypophysial peptides 319

Mark Nickerson—Adrenergic mediators 331

F. C. MacIntosh—Formation, storage, and release of acetylcholine at nerve endings 343

A. S. V. Burgen—Discussion 357